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A REVIEW OF THE INSECT IMMUNE SYSTEM AND EVIDENCE FOR FAD-GLUCOSE DEHYDROGENASE IN HEMOCYTES OF THE MOSQUITO *AEDES AEGYPTI*

A Thesis in

Genetics

by

Douglas R. Lewis

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

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Abstract

A major challenge will soon be facing the world as *Plasmodium* species, the causative agent of malaria, steadily become drug resistant. Additionally, the vector mosquitoes themselves have also developed resistance to conventional control measures. One hope of scientists is to characterize and harness the factor(s) that conveys natural resistance to parasitic diseases, allowing normally susceptible strains of vectors to defeat a microparasite invasion.

In this quest much work has focused on understanding the nature of the insect immune system and the differences between refractory and susceptible mosquitoes. Previous work in *Drosophila* and *Manduca* has shown that an enzyme used for molting, FAD-glucose dehydrogenase (GLD), contributes to the insect immune system. Specifically, this enzyme is required for the killing reaction during encapsulation and hypothesized to produce reactive oxidative free radical species.

The work in this thesis attempts to identify and characterize GLD in the hemocytes of the mosquito *Aedes aegypti*. To my knowledge this is the first report of the localization of a specific immune system enzyme in a mosquito hemocyte. Characterization and eventual manipulation of the insect immune system, possibly using GLD, represents one potential mechanism of disease control through vector immune system alteration.

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Chapter 1

LITERATURE REVIEW OF THE INSECT IMMUNE SYSTEM

Overview of Insects

Insects are the most prevalent organisms found on earth. They play an extremely important role in human life, in terms of crop destruction and the spread of disease. Biting arthropods are responsible for the transmission of many diseases, affecting hundreds of millions of people per year. With the obvious importance of controlling these diseases, much research has been conducted to try to understand the biology of both the diseases and the arthropod vectors.

Insects have evolved an immune system; while not as complex as that of a human, it functions quite efficiently. The insect immune system relies upon cellular and chemical (humoral) defenses to destroy invading organisms. In some strains of insect vectors, these defenses are able to preclude transfer of parasitic diseases, while other strains of the same vector species allow full development of the parasite and pass the parasites on to the source of the insect's next blood meal. This thesis will review many of the mechanisms used by insects to deal with microparasite infections and disease

vectoring, and will examine specific instances where these defenses are being studied in mosquitoes in an attempt to control arthropod-borne diseases.

Infectious diseases transmitted by arthropod vectors account for an enormous amount of the disease load present in the human population. The cost of these diseases in terms of human life, suffering and lost productivity is enormous. In 1992 the World Health Organization estimated that there were 120 million cases of malaria, which resulted in 1 million deaths. The search for a viable control method for these diseases is of obvious importance in reducing the impact of arthropod-borne microparasites upon the human population.

Various control methods are available or have been tried, targeting the various stages of the microparasite's life cycle. Controlling the arthropod vector is an obvious target for attack. However, this approach usually involves the widespread use of chemical agents, causing unacceptable levels of collateral damage to the ecosystem, as evidenced from the widespread damage caused by the use of DDT. There are also increasing numbers of reports of development of resistance in arthropods to currently used control measures.

A second possible area for vector-borne disease control is a vaccination strategy for the human population. This strategy, while environmentally friendly, has yet to be realized. In some cases, such as with trypanosomes, the microparasite is able to present an almost infinite number of surface markers to the host immune system, making a vaccine virtually impossible (Donelson and Turner 1985). An additional complicating factor for this strategy is the ability of some organisms to reside in non-human

reservoirs. If a vaccination were developed it would never be able to eradicate the disease, meaning an ongoing program would always exist.

A third possible control strategy is to target the disease-causing micro-organism while it is in the intermediate arthropod host stage of its life cycle. This approach represents an environmentally friendly control strategy, that could effectively eliminate malaria and other microparasites from all reservoirs. A common characteristic of many disease microparasites is the use of an arthropod to serve as an intermediate host and vector. Using an arthropod vector, the microparasites are able to disseminate from host to host, most often a mammalian species. In the instance of mosquitoes, the discovery of microparasite resistant strains (MacDonald 1962; MacDonald and Ramachandran, 1965) raised a unique avenue for disease control. In the resistant strains, microparasites are unable to complete development and/or migrate to the appropriate location within the arthropod host (usually the salivary glands) to effectively infect a human host during the blood meal. With this idea of microparasite control through vector incompetence in mind, much work has been done to identify and characterize the mechanisms, at both genetic and physiological levels, behind the resistance exhibited by these strains of mosquitoes. Research in this area has focused on the immune system of mosquitoes and the distinction between refractory and susceptible mosquitoes. To date, the answer has not been found; however, much has been learned about the chemical and genetic factors associated with resistance.

Insect Immune System

When faced with an immune challenge, the insect immune system has two primary defensive mechanisms, cellular and humoral (reviewed in Dunn 1986, Hoffman 1995, Hultmark 1993, Lackie 1988). Insects have a population of immune cells, hemocytes, free floating in the hemolymph (blood). The hemocytes respond to invasion in several ways. When the immune challenge is a small foreign particle, the hemocytes are able to phagocytize the foreign material. When the foreign material within the insect is too large to be phagocytized, the hemocytes can encapsulate and melanize the object. The melanization process involves a chemical pathway using catecholamines, derivatives of tyrosine, as substrates and results in the deposit of a dense material, melanin, and the death and crosslinking of the hemocytes around the invading body. Encapsulation often results in the death of an invading organism, as well as the isolation of the organism from the insect. The cells most often associated with this reaction are plasmatocytes (lamellocytes) and granulocytes (Christensen and Forton 1986, Nappi 1984, Ratner and Vinson 1983, Russo et al. 1996). In some organisms the response also appears to have a humoral component (Chen and Laurence 1985).

In a model proposed by Ratcliffe (1993), the immune response can be arbitrarily divided into three phases. The first phase occurs within a few seconds of invasion and consists of recognition, action by constitutive antibacterial factors, and phenoloxidase activation. Activation of phenoloxidase has been implicated in the coagulation of

hemolymph, as a killing mechanism, and in the activation of hemocytes. The second phase lasts from a few seconds to several hours post infection. In this phase, hemocytes actively phagocytize the invading organisms (if they are small), or encapsulate the larger objects. *De novo* expression and synthesis of antibacterial proteins is also observed in the second phase. The third phase lasts from several hours to 7-8 days post infection. In this phase phagocytosis may continue and the synthesized antibacterial proteins continue to fight infection and offer an elevated immune state for the insect (Ratcliffe 1993).

The insect also has a humoral-based defensive system which relies upon antibacterial and antifungal chemical factors, produced primarily in the fat body and in some hemocytes, to assist the cellular response in elimination of infections. There are several classes of these polypeptides (discussed later), and not all are present in each species of insect. On average, the polypeptides are small molecular weight, and most act by disrupting some function of the bacterial membrane. The antibacterial polypeptides can be induced through wounding, bacterial injection, injection of lipopolysacharides, and other chemical inducers.

Cellular Responses

Insects have free floating cells within their hemolymph called hemocytes. Several different cell types are present, the main types being plasmatocytes and granulocytes. These cells offer protection to insects in different ways. Plasmatocytes appear to be the major phagocytic cells present in the hemolymph, while granulocytes

appear to play a helper role to plasmatocytes, stimulating phagocytosis and the encapsulation responses (Anggraeni and Ratcliffe, 1991).

When faced with a relatively small invader, one of the primary responses of hemocytes is phagocytosis. Phagocytosis involves the recognition of foreign material and the subsequent engulfing of the material by the hemocytes. It appears as though phenoloxidase plays an important role in regulating the phagocytic activity of hemocytes. Incubation of hemocytes with beta 1,3-glucans appears to activate the phenoloxidase system and increase hemocyte activity; however, incubation with serine protease inhibitors, which prevent phenoloxidase activation, resulted in little hemocyte activity (Leonard et al. 1985). It also appears that hemocyte cell-cell interaction is needed for increase phagocytic activity. Hemocytes purified from *Galleria mellonella* demonstrate that plasmatocytes alone have a weak phagocytic activity; which increases with the addition of granulocytes. Assays for phenoloxidase activity revealed that only granulocytes exhibit activity (Anggraeni and Ratcliffe, 1991).

Encapsulation of foreign material is one of the most important mechanisms used by insects as a reaction to objects too large to be phagocytized by hemocytes. This process can be divided into two phases. Phase one involves the aggregation of hemocytes around the foreign material. Phase two involves morphological changes of the hemocytes, flattening, and the melanization of the foreign material. The ability to prevent phase two but not phase one with metabolic inhibitors in *Heliothis virescens* implies that phase one does not require metabolic activity, while phase two does (Ratner and Vinson, 1983).

One of the key components in the encapsulation response is phenoloxidase, usually present as a proenzyme to avoid collateral damage to the insect. When activated, phenoloxidase has the ability to regulate several chemical reactions. Two of the reactions utilizing phenoloxidase are the polymerization of proteins and the oxidative polymerization of catechols and phenols.

When hemolymph was removed from *Manduca sexta* under septic conditions and assayed for phenoloxidase activity, the activity was found to be in a complex of a molecular weight much higher than pure phenoloxidase. This complex was not present in larvae bled under sterile conditions. Characterization of the complex has shown that the complex contains several factors, three of which appear to be prophenoloxidase, phenoloxidase and an interleukin-like protein. Such a complex may provide the insect with a recognition/activation mechanism, coupled with a melanization initiating enzyme. (Beck et al. 1996).

Several other enzymes and products have been identified as participants in the melanization cascade. Dopa decarboxylase is an enzyme that converts dopa to dopamine, both intermediate products in the melanization cascade. The importance of this enzyme in the immune system and melanization reaction is observed in temperature sensitive mutants. Infection at a non-permissive temperature resulted in less than 9% encapsulation, while control flies demonstrated over 80% encapsulation.
These results imply that at least some of the material used in melanotic encapsulation is derived from the oxidation of O-hydroquinones and O-quinones derived from tyrosine (Nappi et al. 1992).

However, in insects, the killing mechanism of encapsulation has yet to be discovered. Experiments conducted using *Drosophila melanogaster* strains resistant to the parasitic wasp *Leptopilina boulardi* demonstrates that susceptible strains *of D. melanogaster* can melanize the wasp egg without killing the developing parasite. This experiment did show an increased level of $O_2^{\cdot -}$ associated with the melanization response. Work with mutants defective in catalase and superoxide dismutase demonstrated a successfulmelanization responses but did not kill the wasp embryo. The survival of the wasp embryos indicated that neither $O_2^{\cdot -}$, H_2O_2, or the quinone precursors present during melanization are responsible for parasite death. These results have led Nappi to make several proposals about the killing mechanism used by insects. He speculates that the killing mechanism is a free radical, probably $^{\cdot}OH$, generated from the oxidation of Cu^{2+} to Cu^{+}, which would be tightly associated with an enzyme binding surface. Melanin would then have two functions, to isolate the immune reaction from the rest of the insect and to serve as a scavenger of free radicals to protect the insect from its own immune system (Nappi et al. 1995).

Detailed comparisons of the encapsulation process between *Drosophila* species resistant and susceptible to parasitization by *L. boularidi* have demonstrated differences in encapsulation. Three distinct levels of encapsulation are observed: formation of an electron dense dotted layer, accumulation of hemocytes, and necrosis of inner hemocytes. In resistant strains formation of the dotted layer was observed while susceptible strains did not form such a layer. These results suggest an interaction

between the particles and some component of the insect immune system which is an essential step in the initiation of the encapsulation response (Russo et al. 1996).

Humoral Responses

Humoral responses are manifested as a series of anti-bacterial and anti-fungal proteins produced primarily in the fat body of the insect and by some hemocytes (reviewed in Hoffman and Hetru 1992, Cociancich et al. 1994). More than 50 molecules in several classes have been characterized to date. Cecropins, composed of 35-39 amino acid residues, are effective against most bacteria. They interact with lipid membranes, creating voltage dependent ion channels and breaking down the bacterial membrane. Defensins, or sapecins, are cysteine-containing residues that act primarily against Gram-positive bacteria, slowly killing the bacteria, perhaps through potassium channel blockage. Attacin-like proteins are a class of polypeptides that affect dividing bacterial cells by blocking synthesis of membrane proteins (Hultmark 1993). A class of polypeptides discovered in species of Diptera is diptericin. This class is composed of small polypeptides and is effective against Gram-negative bacteria (Dimarcq et al. 1988). Lysoszymes are another class of proteins some insects use as defensive molecules; these act upon the bacterial cell wall. However, these polypeptides are not used by all insects and are actively suppressed by some species, such as in *Drosophila* upon immune challenge (Kylsten et al. 1992).

Characterization of attacin proteins has given insight into their function, as well as information concerning the bacterial control systems for the expression of membrane proteins. The specific target of attacin appears to be the bacterial membrane. Attacin increases membrane permeability and renders the bacteria more susceptible to other defensive proteins. The attack on the bacterial membrane appears to be through the disruption of membrane proteins OmpC, OmpF, OmpA and LamB, apparently at the level of transcription, although the attacin molecule itself does not appear to be taken into the bacterial cell. This evidence implies that a bacterial control mechanism for membrane polypeptides exists which is influenced by the attacin proteins. The fact that only these four membrane proteins are affected without affecting other surface membrane proteins suggests an alternate bacterial control function exists to control Omp gene expression, which has yet to be described (Carlsson et. al. 1991).

The diptericin class is active against *E. coli* and appear to consist of three basic polypeptides. Characterization of the polypeptides indicates they are a closely related family. They appear to target the bacterial membranes as they are only effective against actively growing bacteria (Dimarcq et al. 1988). Characterization of the diptericin proteins show a proline rich domain (P-domain) and a glycine rich domain (G-domain). The structure of the diptericin proteins suggest a relationship to other classes of antibacterial polypeptides, the attacins and sarcotoxins II (Wicker et al. 1990).

Characterization of the cecropin gene family in *Hyalophora cecropia* shows the cecropin genes exist over a 20 kB stretch of DNA. Three genes are present encoding for cecropins A, B and D (Gudmundsson et al. 1991). Within *Drosophila,* cecropin genes

are expressed in the fat body in response to bacterial challenge. In addition to the fat body, some hemocytes express cecropin as well. In *Drosophila* three genes exist; CecA1, Cec A and CecB. CecB shows preferential expression in the pupal stage and overall expression in the fat body is not as strong as CecA (Samakovlis et al. 1990).

Insects also have a series of agents that exhibit anti-fungal activity. In *Drosophila.* Septic injury induces an antifungal molecule as well as the normal antibacterial molecules. This molecule appears to be synthesized in the fat body, and transcription levels are enhanced with injury. The molecule itself is small and has the potential to form four disulfide bridges. In high doses the molecule completely inhibits the growth of fungi. Lower concentrations will allow fungal growth, but hyphae grown under these conditions grow at a much lower rate and exhibit physiological abnormalities (Fehlbaum et al. 1994). An additional antifungal molecule has been isolated from the flesh fly *Sarcophaga peregrina.* This molecule is constitutively expressed and effective against certain types of fungal infection. Addition of sarcotoxin 1A, an antibacterial molecule, to the media appears to increase the antifungal characteristics of the molecule (Iijima et al. 1993). While it is clear from these experiments that insects have both an anti-fungal system and an anti-bacterial system, the anti-fungal system is much less well studied. The control of and recognition mechanism for this system have yet to be elucidated.

Self vs. Non-Self Recognition

The most basic requirement for an immune system is the ability to recognize "self" from "non-self" or the ability of the insect to determine what material is foreign to the organism. To date an immune "memory" system in insects has not been fully identified in insects. Experiments with the American cockroach, *Periplaneta americana* seem to indicate that a specific immune response exists which is transferable and appears in two sequential stages. Results suggest that a "vaccination" with a bacterial strain will increase subsequent survival of the cockroach when specifically challenged by the same bacterium (Karp and Mead 1993, Karp et al. 1994, Faulhaber and Karp 1992).

Although initial evidence exists for a type of memory in the cockroach, no evidence of such a system has been discovered in other insect species. Without a memory based immune system, insects must rely upon other factors to mediate the immune response to foreign materials. The mechanism insects appear to use for targeting of foreign organisms is the recognition of unique surface molecules conserved among bacterial and fungal species. Such molecules include lipopolysacharides, peptidoglucans, and glucans associated with bacteria and fungi cell membranes and walls (Hultmark 1993).

A molecule that appears to have a role in recognition and immune system activation is hemolin. Hemolin demonstrates the ability to bind the surface of hemocytes and to activate aspects of the immune system. The binding specificity of

hemolin appears to be general in nature. The molecule is able to bind to bacterial strains with mutations in several cell surface lippo-polysacharides (LPS). Increased binding specificity of hemolin may be a result of complexes formed among hemolin, the bacteria and other, more specific insect hemolymph factors which do not bind LPS mutant bacteria (Schmidt et al. 1993).

Hemomucin is another factor that may play a role in immune system recognition. The factor was initially identified in *Drosophila* through hemocyte binding assays conducted with a snail (*Helix promatia)* lectin. Characterization of the protein identified a transmembrane domain, a mucin-like domain, and a domain similar to plant strictosidine synthase. Localization of protein synthesis revealed it was expressed throughout embryonic development and within hemocytes, the gut and ovary of adult flies. While many hemocytes appear to contain hemomucin, only a subset of cells express it on the cell surface. In the rest of the hemocytes, it is stored in intracellular granules, where its surface expression and activation may be controlled by a yet unknown factor. Membrane localization of hemomucin would support one model of proposed immunity in which *Drosophila* soluble lectins would bind to invading microorganisms and link them to hemocytes via membrane bound hemomucin (Theopold et al. 1996).

Control of the Immune System

The control of any biological system is dependent upon communication of physical cues from the environment to the nucleus of specific cells, regulating the

expression of genes to produce the appropriate proteins. While these events form a continuum in the immune response and are tightly integrated, for review purposes it is easier to separate the physical factors affecting the immune response and the genetic mechanisms which respond to these factors.

Physical

The activation of the immune system is based upon input from various sources. Binding of hemocytes and hemolymph factors to components of bacteria and fungal cell walls via hemocyte cell surface receptors would be one method of activation. In *Drosophila* induction of cecropins, antibacterial compounds, is seen using LPS, beta 1-3 glucans and bacterial flagellin (Samakovlis et al. 1992). While introduction of foreign material usually results in physical damage to the insect, damage alone can initiate melanization reactions and expression of antibacterial polypeptides (Brey et al. 1993).

Activation of the immune system requires that a signal be relayed to the insect defensive organs, such as the fat body, for production of the appropriate polypeptides. Transfers of cell-free hemolymph and hemocyte factors from immune activated donors to naive insects demonstrate the potential existence and location of transferable immune factors. Specifically associated with the transfer of hemocyte extract was an increased resistance in recipient insects to lethal doses of bacteria, as well as an increase in lysozyme activity. The transfer of this activity through the hemocyte extract and not the

hemolymph suggests a cell-cell signaling process may be controlled in some part by the hemocyte population (Wiesner 1991).

A realistic challenge faced by insects is damage to the integument through abrasion or attack. While the innate hardness of the cuticle serves as an excellent passive defense, the integument may also take an active role in the immune system. Insects subject to cuticular abrasions of differing severity in sterile and non sterile environments were assayed for the presence of antibacterial compounds. Abrasion of the cuticle in the presence of bacteria resulted in the epidermis producing several compounds belonging to the cecropin family (Lee and Brey 1994).

Differences in the severity of the abrasion also appear to determine the type of immunological activation observed. Sterile abrasions produced no noticeable changes; however, light abrasions (outer cuticle layer, epicuticle, only) in the presence of naturally occurring bacteria produced antibacterial cecropins specifically localized in the cuticle of the abraded area. Deeper abrasions, into the procuticle, produce antibacterial cecropins in the cuticle and in the hemolymph, suggesting a more systemic defensive reaction (Brey et al. 1993).

One factor that plays a role in control of the immune system of insects is hemolin, a 48 kDa protein upregulated in response to bacterial infection. In experiments, addition of hemolin to hemocytes both inhibited aggregation in a dose dependent manner and increased phagocytic activity of hemocytes. Hemolin's ability to suppress aggregation and its inability to disrupt pre-aggregated hemocytes suggest the hemolin may be binding to hemocyte cell surface proteins that are involved in hemocyte

aggregation. Such cell surface binding could indicate that hemolin may be involved in mediating the binding of foreign cells and hemocytes, suggesting a role in the recognition of foreign material. Adding hemolin to hemocytes also caused an increase in protein-kinase C activity, suggesting hemolin may also act as a signal to induce an immune system cascade (Lanz-Mendoza et al. 1996).

Genetic

A similarity of insect and mammalian immune systems that has been demonstrated is that many defensive peptides produced by insects have an upstream region homologous to the NF-*k*B binding region seen in mammals. In mammals the NF-*k*B binding region serves as a recognition region for various types of interleukin for regulation of the initial immune response. Using the binding characteristics and associated factors of the NF-*k*B region, several candidates for genetic control of the insect immune system have been identified, and several of the genetic control regions with their corresponding genes have been identified and characterized.

Attacin genes, found in *H. cecropia,* also appear to be controlled by a NF-*k*B-like domain. Characterization of the attacin locus has identified one acidic and one basic domain translated in opposite directions, as well as the presence of two pseudogenes. The attacin family of polypeptides contains six different species, which are likely to be the products of post-translational modification of products of the two gene sequences. It has been proposed that using post-translational modification the two

gene sequences can account for all six identified polypeptides. Induction of the two genes shows different patterns: the acidic gene expressed in response to injury and infection, and the basic gene is expressed only in response to infection (Sun et al. 1991a).

In *Drosophila* two genes from the *Rel* family, *Dorsal* and DIF (dorsal-related immunity factor), have the ability to bind distinct NF-*k*B-like motifs. Both factors are produced in immuno-associated tissues, are translocated into the nucleus after bacterial challenge, and can initiate production of certain defensive proteins. Evidence also indicates that the two factors can heterodimerize; however, the NF-*k*B motifs recognized by the two factors are distinct and each is not able to efficiently bind the NF-*k*B motif recognized by the other (Gross et al. 1996). In developing *Drosophila* embryos, *toll* plays an important role in the isolation of *dorsal* within the nucleus to act as a dorsal-ventral signal. In *toll* mutants, DIF localization was demonstrated within the fat body; DIF also shows localization to the nuclei of the fat body after a bacterial challenge (Ip et al. 1993). Some *toll* mutants in which *dorsal* is constitutively nuclear demonstrate melanotic tumors, although tumor formation is not *dorsal* dependent. These observations indicate dorsal may play a role in the immune system, although complex interactions and alternate pathways probably also exist (Lemaitre et al. 1995a).

Associated with the cecropin antibacterial response is a nuclear regulatory protein Cecropia Immunoresponsive Factor (CIF) which has demonstrated the ability to bind NF-*k*B like sequences in *Hyalophora cecropia,* much as DIF does in *Drosophila.* The activity and characteristics of these two factors are so similar it is conceivable that

both are the same factor expressed in different species. Both DIF and CIF share the same electorphoretic mobilities and demonstrate the ability to bind both the *H. cecropia* attacin and the *Drosophila* cecropin NF-*k*B-like sequences (Engstrom et al. 1993).

CIF is not normally associated with healthy pupae and is strongly induced upon bacterial challenge. Upon challenge, the appearance of CIF is strongly correlated with the transcriptional activation of immune genes. An additional cytoplasmic factor with a larger molecular weight, C1, which may be the cytoplasmic precursor of CIF, has also been identified. Both factors appear to have the same binding domain although C1 has an additional sub-unit not found on CIF. The sub-unit may be a cytoplasmic factor that binds CIF and keeps it in the cytoplasm to deal with an immune challenge (Sun and Faye 1992a).

CIF is present in the nucleus within 2 hours of an immune challenge and remained present until at least 144 hours post infection. CIF is also present in the cytoplasm, appearing at the same time as observed in the nucleus, although the CIF signal disappeared within 48 hours post infection. It would also appear that CIF is present in the cytoplasm prior to infection. When cells were treated to preclude protein synthesis, CIF was still localized to the nucleus after an immune challenge, indicating CIF was synthesized and present in the cytoplasm prior to the immune challenge (Sun and Faye 1992b).

Work has been done to elucidate the activation mechanism for the antibacterial polypeptide diptericin, a 9 kDa peptide effective against gram-negative bacteria. The diptericin gene is present in a single copy with expression of the gene product appearing

2 hours post infection, peaking at 4 hours and then declines to non-detectable levels (Wicker et al. 1990). Expression patterns differ between an artificial fusion constructed from the diptericin control region and the beta-galactose gene and the native gene based upon insect age, indicating the presence of additional control regions beyond the fusion construct. Expression was detected within the fat body in a cell autonomous manner. Expression within the hemocytes was not detected using the fusion gene (Reicharet et al. 1992).

The promoter region of the diptericin gene contains two 17 bp repeats that share homology to mammalian NF-*k*B sequences. Mutated control regions containing only one repeat show a greatly reduced induction of diptericin production (Meister et al. 1994). Similarly, artificial constructs with reporter genes showed strong activation of the repeat/reporter complex when two or more repeats were used, while activation diminished with the removal of one repeat and was abolished when both were removed. Activity was specifically observed in the fat body. Additionally protein binding was demonstrated after induction with either *Drosophila* protein or mammalian protein p50, a recombinant protein that binds mammalian NF-*k*B sites (Kappler et al. 1993).

An additional antibacterial polypeptide produced by some insects is lysozyme. Characterization of *H. cecropia* has determined that one lysosozyme gene copy exists; although, two mature polypeptides appear to be produced. As with several of the other antibacterial polypeptides, the lysozyme gene also appears to have an NF-*k*B-like binding site in the upstream control region. Induction of the lysozyme gene products is possible using several factors known to induce NF-kB controlled genes. Lysozyme is

present 2 hours post induction and disappears 16 to 24 hours post induction (Sun et al. 1991b).

Following an immune challenge, factors already present in the insect may reduce the amount of thiol (a reducing agent) and increase the amount of reactive O_2 species (H_2O_2 in particular). Using reducing agents and reactive O_2 species it has been demonstrated that increased levels of reactive O_2 induce transcription of CIF controlled genes (discussed later), while the reducing agents tend to repress expression (Sun and Faye 1995). The presence of large amounts of $O_2^{\cdot -}$ species has been clearly demonstrated in the *Drosophila* melanization/encapsulation response (Nappi et al. 1995).

Insect/Parasite Interactions

The interaction between insect and invader is a dynamic one. Just as the insect tries to defeat the presence of the foreign body, the invading organism tries to successfully defeat the insect immune system. From these conflicting goals several unique strategies have emerged on the part of the invading organisms.

Recognition and Avoidance Strategies

As with any evolutionary system, co-evolution leads to the development of strategies by the pathogens to defeat the defensive systems used by insects. Many

strategies can be used by pathogens of insects, although almost all are based upon active suppression of or hiding from the insect immune system.

Several different approaches to defeating the insect immune system have been developed by fungi. To invade *Spodoptera exigua,* the beet armyworm, *Beauveria bassiana* uses two different strategies, avoidance and suppression. Spores and blastospores within the hemolymph appear to repress the immune system by an unknown factor. Repression is evidenced by an overall reduction in hemocyte count, as well as a reduced ability of granulocytes to adhere, form filopodia, and to phagocytize. Additionally blebbing of hemocytes is observed, indicating cell membrane damage. As the invasion progresses, hyphal bodies are produced and are free floating in the hemocoel. Although viable hemocytes are still present, the hyphal bodies are not phagocytized, indicating an apparent avoidance strategy (Hung and Boucias 1992).

Further characterization of the hyphal bodies has demonstrated that, before emergence from the hemocyte, the hyphal body appears to lose its cell wall. Lacking the cell wall, the hyphal bodies are not recognized by lectin probes and apparently are un-recognized by the insect hemocytes. The cell wall does appear later in development, although, by this time, the insect defense system is no longer able to effectively deal with the infection (Pendland et al. 1993).

Polydnavirus

One specific and interesting avoidance system is used by wasps in the families Braconidae and Ichneumonidae. These wasps have incorporated into their genome a virus which is only expressed by the female in the calyx, a region of the ovaries. The virus is injected with the venom and egg during parasitization of a host larva. Within the host larva the virus appears to convey immunity to the developing egg. The virus is transmitted vertically within the wasp. Interestingly, within the parasitized larva transcription of the virus is detected, although no production of viral particles or infectivity of parasitized larvae hemolymph is observed (Strand 1994).

Tracking the viral particles with electron microscopy demonstrated that the viral capsules invaded all types of hemocyte populations. Detection of viral mRNA demonstrated transcription in all cell morphotypes within four hours of parasitization with the greatest expression in the plasmatocytes and granulocytes. The effects of the virus on hemocytes are characterized by a rounded appearance of hemocytes, an inability to spread and adhere, and blebbing (Strand 1994).

Because of the importance phenoloxidase plays in the insect defensive cascades (discussed previously), it serves as a prime target for disruption of a host's immune response. *M. sexta* larva parasitized by *Cotesia congregata* showed a significant decrease in activity associated with the conversion of dopa from tyrosine, a reaction relying upon phenoloxidase. A similar decrease in enzyme activity was not observed due to inactivated virus or venom-only controls (Beckage et al. 1990).

GLD and Its Role in the Immune System

Another enzyme with a major role in the insect immune system is FAD-glucose dehydrogenase (GLD) (EC 1.1.99.10). GLD was first incorrectly characterized as a hexokinase HEX-1 and glucose oxidase. GLD is characterized by the conversion of glucose to gluconic acid with concomitant reduction of the tightly bound FAD cofactor and without the requirement for NAD or NADP. Tissue-specific expression of GLD was first seen in the *D. melanogaster* male ejaculatory duct, and GLD enzyme was observed to be transferred to the female during mating (Cavener 1980).

Further characterization of *Gld* expression demonstrated transient expression in both sexes in the epidermis during the pupal stage of development and with tissue specific expression in reproductive organs of both male and female *Drosophila* species. During larval development, *Gld* expression parallels ecdysteroid levels, strongly suggesting that ecdysteroid acts as a control factor (Murtha and Cavener 1989, Cox-Foster et al. 1990). In all cases the tissues expressing GLD in adult flies were subsets of tissues expressing GLD in pre-adult stages (Schiff et al. 1992). In the pupal stage, null mutants were found to lack the ability to enclose from the puparian cuticle. Rescue of adult flies is possible through manual excision of the end of the puparian capsule (Cavener and MacIntyre 1983). In male flies of several species, a correlation between ejaculatory duct size and GLD expression is observed (Cavener 1985). Characterization of the entire *Gld* gene in several different species of *Drosophila* yielded many

similarities. The copy number and exon/intron structure are maintained in all species studied. It was also demonstrated, through transgenics, that the genes were interchangeable between species (Krasney et al. 1990). Differences in expression patterns have several possible causes, including sex-regulated differences among species and differences in *cis* and *trans* control sequences between species. Evidence for these control sequence differences is seen when *Gld* genes of different species mimic the expression pattern from the donor species (Schiff et al. 1992).

Complementation analyses and chromosomal walks have mapped the *Gld* locus. Chromosomal walks identified a region encoding 2.8 kB mRNA sequence, which hybridized to chromosome site 84B-D and demonstrated expression patterns similar to GLD activity patterns. Qualification of the 2.8 kB mRNA revealed it's presence not only at pupal enclosion but also at other molts, suggesting *Gld* RNA transcription may be under the control of a molting hormone, particularly ecdysterone (Cavener et al. 1986). Sequencing of *Gld* revealed a curious repeat located within the 8 kB intron. The repeat is a dipyrimidine-dipurine repeat named the YYRR box. The repeat is highly conserved among several *Drosophila* species. The YYRR box was found in one other gene, *Ted*, which is tightly linked to the *Gld* gene and demonstrates a similar phenotype. The high conservation of the sequence among several *Drosophila* species suggests that the sequence plays some sort of active role in G*ld* expression (Cavener et al. 1988).

Characterization of the *Gld* control regions has shown that most are found from a -425 to +84 bp relation to the initiation codon. Reporter genes containing this fragment of the promoter region had expression in most tissues normally expressing

Gld. In this promoter region several variations of a TTAGA sequence were identified as the major constituents for controlling *Gld* expression patterns (Quine et al. 1993).

An important discovery about GLD was the determination that the enzyme plays a role in the insect immune system. In *M. sexta* pseudoparasitized with latex implants, GLD levels increased 100-10,000 fold and activity was localized on the implants. The GLD enzyme is localized within the cytoplasm of plasmatocytes and rarely detected within granulocytes. The induction of GLD activity within an immune challenged insect and the biochemical characteristics of enzyme activity has led to a proposed model in which GLD and phenoloxidase participate in an enzymatic pathway that forms semiquinone radicals and oxidative free radicals. These radicals can contribute to the melanization reaction, as well as participate in the killing mechanisms associated with encapsulation, as depicted in Figure 1(Cox-Foster and Stehr 1994).

GLD activity is induced by implants containing bacteria and induced to high levels by implants containing yeast, beta-1-3-glucans or chitin (Stehr 1994). In *Drosophila* experiments with GLD null mutants, P-element transformed mutants with restored GLD, and wild type flies demonstrated that GLD activity is induced in wild type and transformed flies following yeast injections (Cox-Foster, personal communication). Flies lacking GLD activity have significantly higher mortality than wild type flies following live yeast infection (Stehr 1994). Live yeast are recovered from flies lacking GLD and not from wild type (Cox-Foster, personal communication). These experiments demonstrated that GLD is required for survival and the killing mechanism during encapsulation.

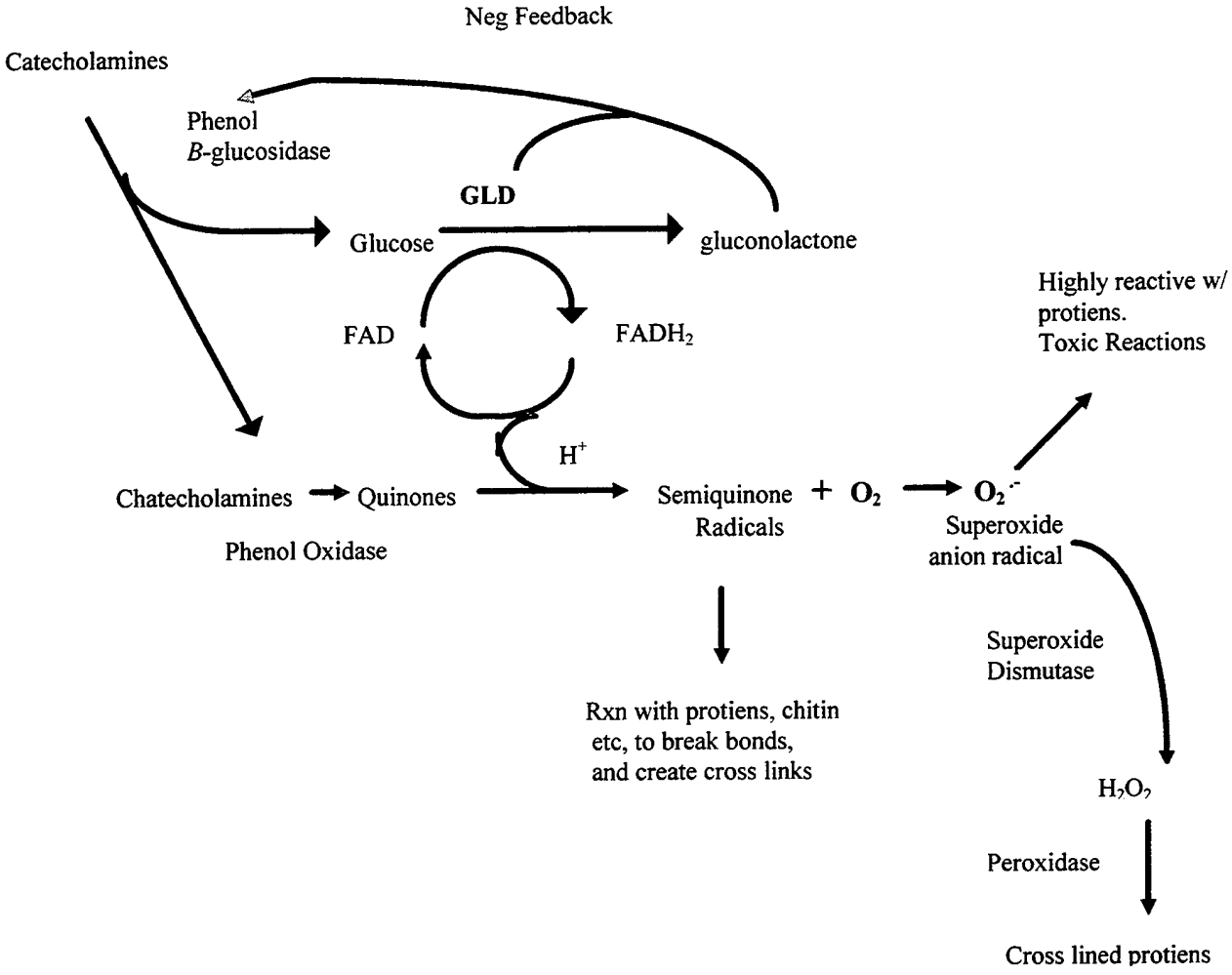

Figure 1. Proposed pathway for GLD involvement during encapsulation. GLD would oxidize glucose to gluconolactone with reduction of FAD. Electron transfer from $FADH_2$ to quinones would create semiquinone radicals. These radicals could be used for bond modification or could react with oxygen to create toxic super- reactive oxygen species (Cox-Foster and Stehr 1994).

Chapter 2

MOSQUITOES AND MICROPARASITES

As insects, mosquitoes share the immune system with the general characteristics reviewed in the previous chapter. However, because the female mosquitoes blood feeds, she can be exposed to organisms not normally encountered by non-blood sucking insects. Several of these organisms are highly pathogenic to mammals and birds and use the mosquitoes as a vector for spreading from host to host. The impact and importance of these diseases on the world human population has funneled much attention to the specifics of the mosquito immune system in an attempt to control the spread of mosquito vectored diseases.

Diseases Transmitted by Mosquitoes

Most disease-causing microorganisms undergo a complex life cycle involving at least two hosts, one often being an arthropod used for transmission of an intermediate parasite life form. In a classic disease such as malaria, the female mosquito draws a blood meal from an infected organism, ingesting gametes which fuse within the mosquito gut to form oocytes (or ookinetes). The oocytes then undergo development and sporulation, with the sporozoites migrating to the salivary glands of the mosquito.

When the mosquito takes its next blood meal, the sporozoites are injected into the blood donor where they migrate to the liver, undergo further development and eventually form gametes to repeat the cycle (Hickman et al. 1986).

Within the host mosquito, the microparasite encounters a variety of environments and tissues as it migrates from the midgut to the salivary glands. To complete this migration, each species may take a different route. For example, when ookinetes from *Plasmodium gallinaceum* penetrate the midgut, some ookinetes appear to go between the midgut cells, while other ookinetes appear to travel through the midgut epithelial cells (Meis and Ponnudari 1987, Torii et al. 1992). While within the midgut cells, the ookinetes are completely surrounded by the host cytoplasm. Electron microscopy has shown that in some host cells an alteration of the cytoplasm takes place. Whether this is a host or parasite induced reaction is not clear (Torii et al. 1992). The multiple life cycle stages and tissues involved in the process present many challenges to researchers but also offer many potential targets for control mechanisms. When the microparasites finally reach to the salivary gland they appear to use a specific targeting mechanism to recognize the glands. Within *A. aegypti, P. gallinaceum* oocytes preferentially invade the distal regions of the salivary gland. Invasion of the gland involves the formation of a vacuole around the sporozoite, which degenerates within the gland only to re-form as the sporozoite exits the cell and migrates into the salivary duct. Interestingly, while within the gland cells a clumping of mitochondria was observed surrounding the sporozoite (Pimenta et al. 1994).

Target recognition is a key factor in the life cycle of most parasitic disease organisms. In each host, specific tissues must be identified by the parasite for the parasite to correctly invade and complete a stage of development. While some of the targeting molecules are most likely native to the parasite, there is evidence that some *Plasmodium* species rely upon the uptake of host factors for successful targeting and binding of cells. In sporozoites pre-incubated in normal mouse serum, lectin binding was observed. However, in sporozoites grown without serum and subsequently exposed to a range of lectins, no binding was observed (Schulman et al. 1980).

Characteristics of Refractory Mosquitoes

The determination of a mosquito's refractoriness to a particular microparasite is based upon the ability of the microparasite to complete all life cycles associated with the mosquito host and be viable and available for successful transmission to the next host (reviewed in Christensen and Severson 1993). The exact measure of refractoriness is somewhat arbitrary; each author may use a different numerical cut-off of viable microparasites to quantify a refractory vs. susceptible mosquito. No matter what system one uses for quantifying refractory and susceptible mosquitoes, it is evident that differences exist among species and strains of mosquitoes in their ability to deal with parasitic invasion. Often refractoriness is not only determined by the species and strain of mosquito but by the strain of the parasitic organism as well. Among mosquito species, it is recognized that some species are inherently more resistant to invasion than

other species as seen when comparing *A. trivittatus* with *A. aegypti* (Li and Christensen 1990).

Defensive Mechanisms of Mosquitoes

As with any insect, the mosquito has several defense mechanisms available to deal with invasive organisms. These methods include the cellular and humoral systems. In addition, due to the blood-sucking nature of the mosquito, some physical barriers are present that present unique challenges to the parasite's life cycle.

The Peritrophic Membrane

The peritrophic membrane (PM) is the first of many physical barriers faced by microparasites in completing their life cycle within the mosquito host. The PM is secreted by midgut cells and is composed mainly of chitin and proteins, with proteoglycans and other sugars present in smaller quantities. Once formed, it completely surrounds and isolates the blood meal within the midgut from the mosquito itself (Shahabuddin and Kaslow 1994).

While the PM undoubtedly plays an important role in impeding the movement of microparasites out of the mosquito midgut, it may not be the primary factor responsible for refractoriness. In experiments where the PM was prevented from forming, mosquitoes had no significant difference in infection rates between naturally refractive

mosquitoes and mosquitoes of the same strain that did not form a PM. This evidence indicates that in refractory species the PM formation is not the determining factor for refractoriness (Shahabuddin et al. 1995).

Since most species of parasites complete development after the PM has been formed (about 20 hours), a mechanism is needed by the parasites to escape from the PM and to enter mosquito tissues. Based upon the chemical makeup of the PM, chitinase would be a desired enzyme to facilitate crossing of the PM by parasites. Initial experiments indicate chitinase production by *P. gallinaceum* ookinetes 15 hours after zygote formation, corresponding to the time of PM penetration (Huber et al. 1991).

Further experiments conducted *in vivo* with mosquitoes demonstrated that additions of allosamidin (a chitinase inhibitor) into an infected blood meal blocked transmission of *Plasmodium gallinaceum* by *A. aegypti* and *Plasmodium falcinarium* by *Anopheles. freeborni.* Infectious ability returned when the PM was prevented from forming while allosamidin was also administered in the bloodmeal, indicating that *Plasmodium* ookinetes use a chitinase to degrade the PM and allow passage into the midgut. When ookinete culture supernate was mixed with mosquito digestive protease, the chitinase activity increased, suggesting that the chitinase exists as a proenzyme and is modified by a mosquito protease. Further analysis indicates that activation is due to cleavage of a lysine residue (Shahabuddin et al. 1993).

Given the existence of the PM and the need for microparasites to maintain an active mechanism to pass through the membrane, the PM may serve as a potentially useful tool in the control of mosquito borne disease, as has been demonstrated in

experiments using antibodies. Administration of C5, a monoclonal antibody raised against a surface protein of *Plasmodium gallinaceum,* resulted in the inability of ookinetes to cross the PM. No difference was detected between antibody-treated and control *P. gallinaceum* in completion of any other phase of the microparasite life cycle, except for the inability of antibody treated ookinetes to cross the PM and invade the midgut epithelial cells (Sieber et al. 1991). Altering the parasite's ability to cross the PM may be possible through the use of antibodies which could bind to the prochitinase or chitinase, neutralizing the enzymatic activity needed to break the PM. These antibodies could be included in the blood meal through human vaccination, or through transgenic approaches creating mosquitoes expressing antibodies, preferably under control of digestive gene control elements.

Midgut Epithelial Cell Responses

Once past the PM the microparasites must penetrate the cells lining the midgut to reach the hemolymph and migrate finally to the salivary glands. The ookinetes appear to enter in the microvilli of the midugt cells and proceed through the cells towards the basal lamina. The cell cytoplasm surrounding the ookinetes appears devoid of organelles and takes on a fine granular appearance (Torii et al. 1992).

Once the microparasites are through the midgut they are exposed to the mosquito immune system and can be recognized and attacked. In addition to encapsulation and melanization, a novel response has been observed in which

microparasite ookinetes are killed without melanization. Experiments using *An. gambiae* resistant to *Plasmodium gallinaceum* demonstrated a mechanism of mosquito resistance in which the ookinetes migrated into the mosquito midgut epithelial cells but died within the cells. There was no evidence of melanization associated with encapsulation but a change in morphology of the mosquito epithelial cell was noted. Within the cells, dead ookinetes were surrounded by a layer of cytoplasm, which was granular and had filamentous material present. After this organelle free layer had formed, the ookinetes were eventually broken up within the epithelial cell. Interestingly the genetic loci responsible for this trait mapped to the esterase locus *Est A* on the 2La inversion, a locus which has also been associated with a successful encapsulation response demonstrated by a strain of *An. gambiae* refractory to *Plasmodium cynomolgi* B. (Vernick et al. 1995).

Cell Mediated Responses

Once through the midgut, the microparasites are in the insect's hemocoel and are exposed to the hemolymph. From here, the microparasites will migrate towards the salivary glands to complete their life cycle. Within the hemolymph, the microparasite are exposed to the same defensive mechanisms possessed by any insect.

Areas within the hemolymph that provide potential mechanisms for microparasite resistance include the hemocytes. Work by Zhao et al. (1995). has demonstrated changes in the hemocyte population of immune-challenged mosquitoes. In mosquitoes faced with a microparasite challenge, hemocyte levels decrease upon

infection then return to control levels, and the percentage of hemocytes binding wheat germ increases. Additionally, phenoloxidase activity in the immune challenged insects showed a significant increase 12 hours post infection. These results seem to suggest that the mosquito hemocytes can recognize the microparasite invasion and initiate an immune reaction (Zhao et al. 1995.).

However, not all mosquito species respond in the same manner. *Armigeres subalbatus* rapidly melanizes the invading filarial nematode *B. malayi,* has a rapid decrease in hemocyte numbers, and then returns to normal levels of hemocyte populations. In contrast *A. aegypti* takes several days to melanize *B. malayi* and the hemocyte population observed within this species shows a gradual increase over a three-day period (Guo et al. 1995). The source of the new observed hemocytes appears to be mitotic division (Christensen et al. 1989). Throughout the literature concerning hemocyte reactions, different experiments have reported different behaviors of hemocyte populations. In part, this may be due to the difficulty associated, with harvesting mosquito hemocytes and the involvement of hemocytes in the melanization reaction, removing them from circulation.

When levels of monophenoloxidase (MPO) were assayed in immune challenged *A. aegypti,* an increase in MPO was observed. Furthermore, the enzyme was observed in plasma and hemocytes, with the increased levels of enzyme observed in the hemocytes of mosquitoes undergoing a melanotic reaction (Li et al. 1989.). Similar experiments conducted with *Aedes trivittatus* showed an increase MPO three to four times higher than the levels associated with an *A. aegypti* response. As in other

experiments MPO appears to be a product of hemocytes in response to melanization (Li et al. 1989, Guo et al. 1995).

While phenoloxidase is undoubtedly an important enzyme in mosquito refractoriness, the presence of the enzyme does not appear to be the determining factor for resistant mosquitoes. Assays for PO activity showed that, after an infected blood meal, PO activity was present at higher levels in refractory mosquito basal lamina, but significantly reduced or missing in susceptible strains. These results suggest several possible reasons for refractoriness: inability of susceptible mosquitoes to recognize the microparasites, suppression of PO activity in susceptible mosquitoes, or an altered regulation pathway in refractory mosquitoes (Paskewitz et al. 1989.)

Evidence for the direct involvement of hemocytes in melanization has been gathered through microscopic studies of the melanization event. In *A. aegypti* melanization of *D. immitis* microfilaria is characterized by close association of hemocytes with the parasite. Dark granules are observed within hemocytes in close association with the foreign material, followed by apparent lysis of the cells, as evidenced by the presence of cellular debris around and within the melanin coat. As cells lyse, a melanin coat is established around the foreign material. The final step in the reaction appears to be a double membrane-like structure which forms around the melanized parasite, isolating it from the insect environment (Christensen and Forton 1986). This same series of events (hemocyte lysis, followed by melanin production, ending with the establishment of a double layered membrane) was also observed in *Aedes trivittatus* (Forton et al. 1985). However, this process may not apply to all

mosquito species. Work with *Anopheles quadrimaculatus* encapsulation of *Brugia pahangi* suggests that an inner melanin layer is formed without cell lysis or involvement, followed by an outer layer of hemocytes. In these experiments the encapsulation reaction started within 10 minutes and was first characterized by the formation of an evenly distributed electron dense material. This material was free of cellular debris, and it was not until 2 hours later that cellular involvement in the reaction was noted (Chen and Laurence 1985).

Observable physical changes in the hemocyte population are also noted in immune challenged mosquitoes. *A. aegypti* hemocytes from naive mosquitoes demonstrate only a 3.5% - 5.5% ability to bind wheat germ agglutinin (WGA), a test that demonstrates the ability of hemocytes to recognize and bind foreign material and lectins. In immune challenged mosquitoes, the percentage of hemocytes demonstrating WGA binding ability rose to 44% (Nappi and Christensen 1986). The mosquito *A. trivittatus* is able to melanize parasites much more quickly than *A. aegypti*. Similar WGA experiments conducted between the two species showed that the *A trivittatus* hemocyte population from non-challenged mosquitoes had a 30-40% innate WGA binding ability. This high state of "readiness" in *A. trivittatus* hemocytes may explain the rapid melanization of parasites demonstrated by *A. trivittatus* when compared with *A. aegypti* (Li and Christensen 1990).

Humoral Responses

After the cellular responses, another defensive mechanism within the hemocoel of the mosquito is the humoral defenses. Injection of the antibacterial compounds magainin from *Xenopus laevis* and cecropin from *Hyalophora cecropia* arrest the maturation of *Plasmodium* oocytes. While these particular compounds are not from a mosquito species, induction of similar compounds could provide one avenue to induce mosquito immunity to microparasite infection (Gwadz et al. 1989.). Work with cultured mosquito cells has led to the production of a cecropin-like protein, demonstrating that these chemicals are present in the mosquito and could play a role in the overall control strategy (Hernandez et al. 1994).

In some mosquito species it may be that humoral factors may also play a role in the encapsulation response. In *Anopheles quadrimaculatus* it appears that the initial melanization reaction is humoral in that a layer of dark material is seen surrounding the foreign material prior to the observed appearance of hemocytes (Chen and Laurence 1985).

Chemistry Associated with Immune System Chemical Reactions

Melanization is a response mounted by mosquitoes in response to infestation with microparasites. One model of the melanization reaction places tyrosine at the top of the cascade, which is first converted to dopa, which is followed by a series of

quinones ending with the formation of the melanotic capsule. Several enzymes are thought to play a role in the cascade, with phenoloxidase initiating the reaction. The mosquito *Armigeres subalbatus*, which naturally encapsulates *B. malayi,* was used in assays conducted to test for tyrosine and its derivatives along with catecholamine-metaboilizing enzyme activities. Results showed a significant increase in tyrosine and dopa levels after immune activation as a result of blood feeding. Other significant findings showed that tyrosine is depleted as a result of wound healing and that phenoloxidase and dopa decarboxylase activity increase in immune activated mosquitoes (Zhao et al. 1995).

Studies of insect cuticle and chorion can prove useful because both cuticle chorion and the encapsulation tissue contain melanin. Studies of egg chorion tanning in *A. aegypti* once again show tyrosine conversion to dopa via phenoloxidase. The dopa can then be decarboxylated, by dopa decarboxylase to form dopamine. Both dopa and dopamine can then be converted into their quinone forms via phenoloxidase (Li and Christensen 1993).

As with other insects, phenoloxidase plays an essential role in the melanization reaction in mosquitoes. Measurements of cell-free hemolymph from immune challenged mosquitoes show PO activity in immune challenged mosquitoes to be twice the levels observed in the control mosquitoes (Nappi et al. 1987). Activation of phenoloxidase in *A. aegypti* has been shown to depend upon Ca^{++} ion concentration. Evidence indicates that activation is also dependent upon a serine protease with trypsin-like activity. This activity is needed to convert prophenoloxidase to phenoloxidase and

initiate the cascade (Ashida et al. 1990). Interestingly, esterase, a marker which has been associated with refractoriness, may exhibit serine hydrolase activity (Vernick and Collins 1989). In subsequent steps of the melanization cascade, dopa is oxidized in reactions controlled by phenoloxidase. Phenoloxidase appears to have a tyrosine hydroxylase activity and an ortho-diphenoloxidase activity (Li et al. 1994).

In refractory strains of *An. gambiae, Plasmodium* ookinetes are encapsulated and melanized. Assays for tyrosinase phenoloxidase detect activity only after the immune system has been activated, indicating that the enzyme plays a role in melanization and that it exists in an inactive form prior to immune activation. Localization using gold-labeled enzymes pinpoint the salivary glands and the apical granules of midgut columnar cells. Localization of phenoloxidase in two strains of *A. gambiae*, refractory and susceptible, showed identical distribution patterns, indicating that, for this species, the tyrosinase-type phenoloxidase is probably not the factor responsible for refractoriness (Brey et al. 1995).

Several other chemicals have been linked to melanization reactions. Dopa decarboxylase (DDC) has shown tissue-specific activation of the enzyme in the ovaries in response to blood feeding. Lower levels of DDC were also observed in the head, probably associated with neurotransmitter activity, and in the fat body (Ferdig et al. 1996). Considering the similarity between the materials and chemistry involved in egg chorion formation and melanization used as a defensive measure, it is would stand to reason that DDC may be a player in insect immunity. Experiments conducted in

Drosophila showed that DDC mutant *Drosophila* were immune challenged (Nappi et al. 1992).

In addition to these specific observations relating to mosquitoes there is no reason to believe that the proposed superoxide radical involvement in the killing mechanism (Nappi et al. 1995) does not take place in mosquitoes. To date, however, the appropriate mutants have not been identified to reproduce the experiments done by Nappi and colleagues in mosquitoes.

"Passive" Defenses by Mosquitoes

While not an active form of defense employed by the host mosquito, target-tissue recognition by the microparasite plays an essential role in successful completion of their life cycle and is therefore a key factor in refractoriness. While the microparasite is undergoing its life cycle within the mosquito and within the mammalian host, it must target specific tissues at specific points in the life cycle to continue development. One apparent contributor to refractoriness is a disruption in the ability of the microparasite to recognize the appropriate tissue within the mosquito and to complete development. Experiments using *Plasmodium knowlesi* demonstrated that the *Plasmodium* could develop within the midguts of *Anopheles dirus*, its natural host, and within *Anopheles freeborni*, a refractory species. However, transplantation of salivary glands showed that the sporozoites would not or could not invade the salivary glands of *An. freeborni*, but salivary glands from *An. dirus* were invaded when transplanted into infected *An.*

freeborni (Rosenberg 1985). This experiment suggests a presence and involvement of targeting molecules specific to the salivary glands that are recognized by the *Plasmodium* parasites.

The timing of a parasitic infection may also play an important role in the successful transmission of the disease. Using *B. malayi* microfilaria as an immune challenge, it was shown that two day old microfilaria were unable to successfully transverse the mosquito midgut wall. Microfilaria aged beyond one week had an increasing success rate at midgut penetration. Analysis of microfilaria membrane binding to a series of antibodies demonstrated a change in antibody binding associated with cell membrane receptors, suggesting a change in the parasite's outer membrane as it matures (Fuhrman et al. 1987).

Genetics Associated with Refractoriness

Of particular interest to scientists looking for ways to control the spread of mosquito-borne diseases is the genetics associated with refractoriness. Discovery of the factor(s) that control refractoriness will give scientists an extremely powerful weapon against vector-borne diseases. To this end much effort has been directed and several advances have been made.

Identification of Resistance Loci

Subsequent to the initial discoveries of refractoriness in mosquitoes, several studies have further refined the genetic basis for resistance in several mosquito-parasite combinations and have identified several loci and inheritance patterns. Early characterization of *A. aegypti* resistance to *P. gallinaceum* suggested that resistance was a single recessive trait, located on the second chromosome (Kilama and Craig 1969). In subsequent experiments with a refractory strain of *An. gambiae* and two strains of *Plasmodium,* two loci were identified. One locus is tightly associated with an esterase locus, while a second, unlinked locus is the major contributor to resistance to other strains of *Plasmodium* (Vernick et al. 1989).

While each species appears to have unique genetics associated with specialized resistance to parasite species, in many species it appears that several genetic loci contribute to resistance, often with one major locus and several other loci making smaller contributions. Attempts to isolate a strain of *A. aegypti* refractory to *P. gallinaceum* demonstrated that selection by breeding pooled progeny based upon refractoriness did not work. However, isofemale selection was able to produce and maintain a totally refractory and partially refractory line within only a few generations. The maintenance of an intermediate line, as well as a chi-square analysis which shows only an acceptable fit to the model if incomplete dominance is assumed, argue against a simple single locus. Two possibilities that would explain the data are a major control

locus exhibiting variation in expression, or a major locus with several additional minor loci (Thathy et al. 1994).

Locations of Loci

Experiments to determine the nature and location of mosquito resistance have given clues as to the underlying genetics of this phenotype. Evidence has shown that resistance to two different filarial parasites, *Brugia malayi* and *Dirofilaria imitis*, can occur in the same strain of mosquito. When *A. aegypti* mosquitoes were challenged with *B. malayi* parasites, seven novel polypeptides were produced by the resistant strains compared to susceptible strains. When challenged with a *D. imitis* parasite, the seven polypeptides were not observed, suggesting that these polypeptides may be important in the immune reaction against only *B. malayi*. If this is the case, these data would suggest that resistance to different parasites involves different loci and that resistance can be controlled and is not a systemic "anti-parasite" system or gene (Watam and Christensen 1992a). Resistance can be further localized to the tissue level. Studies using transplants of Malphigian tubules from *A. aegypti* strains refractory to *D. imitis* demonstrated that factors conveying resistance to this parasite were limited to the Malphigian tubules. Also of interest from this study was the observation from use of transplanted tissues that the resistance is determined by the donor tissue, not the recipient insect (Nayar et al. 1988).

Of great importance in genetic research is the determination of the location of resistance loci in the mosquito genome. Restriction fragment length polymorphism (RFLP) markers represent one mapping technique that can be used to identify loci that segregate with the resistant phenotype. Use of RFLP markers has identified two sites within the *A. aegypti* genome that segregate with resistance to *B. malayi*. These markers map to chromosome one and two and exhibit epistasis, with the locus on chromosome one being a recessive trait, but playing the major role in resistance, and the locus on chromosome two playing a lesser additive role on the effects of the primary locus (Severson et al. 1994).

Additional use of RFLP markers has located other loci relevant to *A. aegypti* parasite resistance to *Plasmodium gallinaceum:* one locus on Chromosome 2 and the other on Chromosome 3. Unlike the loci on chromosomes 1 and 2 conveying resistance to *B. malayi* (Severson et al. 1994), no epistasis is seen between these two loci, and locus 2 accounts for the greatest amount of parasite resistance. The resistance loci on chromosome 2 to these different parasites appear to be in the same location as loci linked to resistance to the yellow fever virus. The close location of many resistance traits to the same area of the second chromosome may indicate a complex locus responsible for several types of microparasite resistance (Severson et al. 1995).

In a study of refractoriness aimed at establishing the number of microfilarial parasites ingested by *A. aegypti* during the blood, meal an additional quantitative trait locus was discovered, affecting the number of microparasites ingested. In refractory mosquitoes, fewer microfilaria (*Onchocerca volvulus*) were ingested, subsequently

affecting the number of parasites able to leave the midgut and to migrate to another site contributing to refractoriness, the salivary glands. Using RFLP analysis, this phenotype (*idb*) was mapped to a locus in the second chromosome (2,LF181) with additional genes suspected to influence resistance of midgut cells to penetration. As a whole, this locus mapped in the same area as loci conveying resistance to *B. malayi* and a locus associated with resistance to yellow fever (Beerntsen et al. 1995).

In the species *Anopheles gambiae* two esterase markers appear to segregate with refractoriness to *Plasmodium cynobolgi*. Each marker is associated with two different alleles encoding distinct polypeptides (Vernick and Collins 1989). Once again, these loci map to an area of the second chromosome. In this instance, however, resistance appears to be related to an inversion of the left arm of the second chromosome (2 La), which appears to repress recombination and maintain the esterase/ refractoriness linkages (Crews-Oyen et al. 1993).

Manipulation of Refractoriness

Once the nature of refractoriness is understood, novel control strategies for disease control can be engineered. Working with the insect's natural immune system components of cellular and humoral components, as well as the mosquito unique aspects, several different targets are present for control of mosquito borne diseases (reviewed by World Health Organization report of the meeting "Prospects for malaria control by genetic manipulation of its vectors," 1991).

Defensive Molecules

Three defensive proteins have been characterized during the mosquito response to bacterial invasion. While defensin activity was found primarily against gram-negative bacteria, there is a possibility that these defensins may function against microparasites (Lowenberger et. al. 1995). Work in this area has shown that *A. aegypti* mosquitoes which have been immune challenged by *E. coli* injections do respond with defensins. Subsequently, if the mosquitoes fed on a microparasite infested host (*B. malayi*), the number of microparasites within the mosquito is significantly reduced as compared to non-challenged mosquitoes. The immune effect from these defensins does show an upper limit; no significant reduction in parasites was observed when mosquitoes took a blood meal from a highly infected host. The effects of these defensins are not the equivalent of the innate immunity used by refractory strains, though, as none of the observed mosquito strains produced defensins as a response to blood feeding from an infected host (Lowenberger et al. 1996).

Novel Proteins

The nature of refractoriness among strains within the same species suggests that the refractory mechanism might be a novel factor unique only to refractory strains. To date, no refractory factor has been identified and characterized. Ongoing work has identified several potential factors.

One specific polypeptide has been isolated which appears to be involved in mosquito refractoriness to filarial worms that are encapsulated. *A. aegypti* mosquitoes were presented with larvae of *Brugia pahangi,* which were treated to either invoke or forgo melanization. In mosquitoes undergoing a melanization encapsulation reaction, a novel 84 kD polypeptide was detected within the hemolymph. Low levels of this polypeptide were observed in mosquitoes undergoing a melanization reaction associated with wound healing, while no increase of the polypeptide was observed in insects not undergoing a melanotic encapsulation response to the parasites. Subsequent sequencing and characterization of the polypeptide revealed it to be basic and probably not a heat shock protein or an antibacterial compound; however, no further insight has been developed into its nature or function (Beerntsen and Christensen 1990, Wattam and Christensen 1992b).

Searches for the 84 kD polypeptide in other species have met with mixed results. Probing of *A. subalbatus* with an *A. aegypti* probe containing the sequence encoding the 84 kD protein demonstrated binding. However, antibodies raised against the 84 kD protein failed to detect the presence of the polypeptide in *A. subalbatus*. These mixed results are possibly due to similar non-specific DNA sequence binding, a different conformation of the polypeptide, or a lack of expression in *A. subalbatus* (Guo et al. 1995).

Other studies with *A. aegypti* refractory to *B. malayi* oocytes isolated six novel polypeptides that were present in the thoraces (the area invaded after the oocytes leave the midgut) of refractory mosquitoes that had been fed a blood meal and were not

present in those fed a sucrose diet. One additional polypeptide was seen in refractory strains fed blood and sucrose, but none of the polypeptides of interest were observed in susceptible strains. These studies indicate that the presence of a blood meal, alone void of microparasites, may be enough to trigger the immune response. Also of interest is the tissue-specific phenomenon of refractoriness. Initial expression of these polypeptides was observed in thoracic tissue only and not in the hemolymph (Wattam and Christensen 1992b).

Peritrophic Membrane

Using the knowledge gained about the microparasite's strategy to cross the PM, different control methods have been tried or hypothesized. Breaking any of several key processes of chitinase production, activation, or action would prevent the parasites from crossing the PM, thus constituting a successful control measure. Trypsin has been identified as potential factor needed to activate chitinase and, thus, would make a good control factor. To verify the role of trypsin, and test for a possible control measure, mosquitoes were fed from an infected blood meal which included trypsin antibodies. In the mosquitoes that received the antibodies, the microparasites were unable to cross the matrix, keeping the mosquito parasite-free (Shahabuddin et al. 1996). While this finding is a long way from a vaccine, it does present an interesting approach to a possible control measure.

Microparasite Targeting of Host Cells / Antibody Mediated Refractoriness

With the multiple cell types and environments encountered by the microparasites, the microparasites must seek out the appropriate host cell to migrate through or to invade. This strategy necessitates that the microparasites be able to recognize target cells, quite possibly by cell surface markers. Lectin binding experiments have shown each area of the *A. aegypti* salivary gland basal lamina exhibits a unique subset of lectin binding patterns (Perrone et al. 1986). Several experiments have demonstrated that altering host cell surface markers can influence microparasite behavior. When *Anopheles tessellates* mosquitoes ingested *Plasmodium vivax* gametocytes, as well as rabbit anti-sera raised against *An. tessellates* midgut tissue, the infection rate of the host mosquito was significantly reduced. However, the effect was only seen during microparasite development within the midgut, suggesting that the antibodies were functioning by blocking the passage of the microparasites through the midgut (Srikrishnaraj et al. 1995).

Similar experiments were conducted using antibodies raised against *A. aegypti* salivary glands and lectins that exhibited binding to the basal membrane of the salivary glands. In this experiment the polyclonal antibodies had specificity for the basal membrane of the salivary glands. Mosquitoes were fed blood meals containing both *Plasmodium gallinaceum* and the antibodies. Upon development the sporozoites did not invade the salivary glands of the mosquito, rendering the mosquito refractory to the microparasites. Seven lectins were identified that showed binding abilities with the

basal membrane of the salivary glands. The use of the lectins in place of the antibodies showed similar results as far as blocking sporozoite invasion of the salivary glands (Barreau et al. 1995). This evidence also supports the hypothesis that the microparasites must identify the target tissue of the host and are unable to complete their life cycle if the target cell signals are blocked.

Other instances of antibodies being used as blockers of transmission include an antibody raised against a 21 kDa protein found on the surface of the rodent malarial parasite, *Plasmodium berghei* (Winger et al. 1988). Antibodies raised against p70 and p75 surface molecules of *Brugia malayi,* a parasitic nematode, have also prevented infection of the vector. Characterization of the surface molecules targeted by the antibodies showed they had a chitinase activity. While *B. malayi* parasites do not face the PM barrier that malarial parasites must overcome, because of the timing associated with parasites leaving the midgut prior to the PM formation, chitinase appears to be important for *B. malayi* to complete its life cycle (Fuhrman et al. 1992). An experiment using antibodies raised against circumsporozite proteins of *Plasmodium gallinaceum* successfully blocked the migration of microparasites to the mosquito salivary glands, apparently by blocking a signal used for the recognition of the glands (Warburg et al. 1992).

Costs Associated with Refractoriness

Most species of mosquito refractory to microparasites use an active defense against the parasitic organism. Melanization is often a key factor in this defense. With the chemical evidence indicating that tyrosine plays a key role in melanization, studies were conducted to assess the impact of refractoriness upon reproductive costs. Tyrosine and melanin formation are also related to egg maturation and chorion tanning. When compared to non-immune challenged mosquitoes, mosquitoes mounting a successfulmelanization defense against microparasites had an increased time to egg laying, smaller ovaries, and a reduced amount of tyrosine in the ovaries (Ferdig et al. 1993).

This raises several questions about the evolutionary advantages of resistance and the future use of naturally refractory mosquitoes for disease control. Resistance to different types of microparasites can mean selective advantage or disadvantage to the mosquito depending upon the parasite type. Filarial worm parasites cause much damage to the tissues of the mosquito host, and infections often result in increased host mortality. A moderate level of refractoriness to filarial worms helps to reduce, but not eliminate, the parasite load upon the vector. This benefits both organisms, the vector is able to withstand the infection, which allows the parasite greater opportunity for dissemination (Ferdig et al. 1993).

However, a level of refractoriness is not advantageous to a malarial type disease. The malaria parasites do not cause much damage to the vector and do not significantly

increase host mortality. Therefore, refractoriness to these parasites does not increase the vector's survival rate, but does put it at a disadvantage in egg laying. The implication of this cost for genetic researchers is that species engineered to be naturally refractory to disease causing parasites must be given a selective advantage which will allow them to out-breed the non-refractory, reproductively efficient species (Ferdig et al. 1993).

The Future of Control Measures

The eventual goal for scientists researching the mosquito immune system is to identify the factors imparting refractoriness and the genetic mechanisms that control them. When this goal is achieved, the next objective will be to transfer these characteristics into a susceptible strain of mosquito. In addition to receiving the refractoriness factor(s) these mosquitoes will need to be given an advantage that will allow them to out-breed all native, susceptible strains of mosquitoes. When this is achieved the world will still be full of mosquitoes that bite. However, they will not transmit the diseases they now carry (WHO 1991, Ferdig et al. 1993).

Several obstacles must be overcome before this goal is reached. The first is the identification of the refractoriness factor(s). Work is continuing in this field and success will most likely be realized. The most daunting task, however, is to develop a method for genetic manipulation of mosquitoes (reviewed in Carlson et al. 1995). The ability to use transposon-mediated genetic manipulation has allowed for extensive work to be accomplished in *Drosophila*. Attempts have been made to manipulate mosquitoes using

P elements, however; the recombination events which were observed appear to be independent of the P element (Miller et al. 1987). Some success has been seen in microinjection of eggs and plasmid integration into cell cultures, but these methods are unreliable and tedious (Carlson et al. 1995).

To date significant advances have been made in characterizing the mosquito immune system. Many of the pathways and components of the immune system have been characterized; although, the specifics of refractoriness have yet to be discovered. One area which has not been examined in much detail is the content of mosquito hemocytes. The next chapter describes some of the first enzymes with an immune function to be localized in mosquito hemocytes.

Chapter 3

IS GLD PRESENT IN *AEDES AEGYPTI?*

Introduction

Understanding the insect immune system is of great importance to many people, given the ability of mosquitoes to vector parasitic diseases. The World Health Organization reported 120 million cases of malaria in 1992, one million of which resulted in death. The emergence of drug resistant strains of *Plasmodium* and insecticide resistant strains of mosquitoes will mean an increase in the number of cases worldwide. To combat this increase, health workers will have less weapons in their arsenal due to the developing resistance.

Work by several researchers has demonstrated that certain strains of mosquitoes exhibit a natural refractoriness to parasitic diseases (Christensen and Sutherland 1984; Kilama and Craig 1969; MacDonald 1962). Knowing that refractory strains exist, much work has been devoted to finding the source of the refractoriness. This knowledge may eventually allow the manipulation of the mosquito genome, allowing conversion of mosquitoes to a phenotype refractory to microparasites in efforts to control disease transmission.

One contributor to the insect immune system is FAD-glucose dehydrogenase (GLD), originally identified in the molting fluid and reproductive organs of *Drosophila* species (Cavener 1980). Subsequent studies of GLD have revealed its presence in the hemolymph (Zheng 1991) and hemocytes (Cox-Foster and Stehr 1994). Characterization of GLD activity in the hemolymph revealed a possible role of GLD participation in the immune response. Increased levels of GLD were observed in insects undergoing an immune reaction to implanted latex. Specific GLD activity was associated with plasmatocytes, and on rarely detected in granulocytes. Also proposed was a chemical pathway in which GLD would act in concert with phenoloxidase to produce semi-quinone radicals and oxidative free radicals, which may be used as in melanin production or as killing molecules by the insect (Cox-Foster and Stehr 1994). Elevated GLD activity is also seen following a yeast infection (Stehr, 1994) and is required for the killing reaction (Cox-Foster, personal communication).

Given the importance of understanding the mosquito immune system, work was undertaken in this thesis to test the hypothesis that the mosquito *A. aegypti* contained gene(s) homologous to *Gld* and that GLD activity would be associated with the immune system, specifically the hemocytes. The underlying assumption for this hypothesis is not unfounded in that GLD activity has been reported in arthropods as far removed from *Drosophila* as crayfish (Cox-Foster and Stehr, personal communication).

Materials and Methods

Mosquitoes

Aedes aegypti eggs (a gift from Dr. Ann Fallon, Department of Entomology, University of Minnesota) were hatched under vacuum in MQ water (Water purified using a Millipore "milli-Q uv Plus" filtering system). Larvae were fed powdered rat chow, sprinkled on top of the water. Larvae were maintained in growth containers at 25 °C. Adult mosquitoes were maintained in wire mesh insect breeding cages (BioQuip Products, Gardena CA) and sustained on a 5% sucrose diet.

Bleeding techniques

Hemocytes were obtained by clipping the head of the second or third instar larva while it was suspended in either *Aedes* saline (Hayes 1953) or Grace's Insect Media (with L-glutamine; without lactalbumin hydrozylate, yeastolate and insect hemolymph) (Quality Biological Inc, Gaithersberg MD). An alternate method employed was to rip apart the larva with forceps while it was suspended in Grace's Insect Media. Obtaining significant numbers of hemocytes was difficult due to the low number of hemocytes per individual. Several larvae (4-6) were combined per slide for increased hemocyte numbers.

DNA Extraction (Nuclear Isolation Method)

For nuclear DNA isolation, approximately 30 larvae were rinsed in MQ water, then frozen in liquid nitrogen. Frozen larvae were then crushed by pestle and homogenized in Nuclear Isolation Buffer (NIB) (10 mM Trizma base (Sigma Chemicals, St. Louis MO) pH 7.4, 60 mM NaCl, 10 mM EDTA, 0.15 mM spermine, 0.15 mM spermidine, 0.5% Triton X-100) and centrifuged at 7,600 g for 7.5 min. The resulting pellet was twice washed in NIB. After the final wash, pellets were resuspended in NIB with 10 % Sarkosyl (sodium salt of N-Lauryl Sarcosine) and set on ice for 10 min. After sitting on ice, the DNA was ethanol precipitated (1/4 vol NH_4OAc, 2.5 vol 100% ethanol) then phenol/chloroform extracted (1 vol phenol; 1 vol phenol / [chloroform/isoamyl alcohol {24:1}]; 1 vol chloroform/isoamyl alcohol) and resuspended in 100 ulTE (10 mM Tris, 1 mM EDTA pH 8.0).

Total Nucleic Acid Extraction

For total nucleic acid extraction (Bingham et al. 1981) approximately 30 larva were rinsed in Quatricide and MQ water and then homogenized on ice in 200 ul solution A (0.1 M Tris, pH 8.0; 0.06 M NaCl; 5% sucrose; 0.01 M EDTA; water up to 50 ml). Then 200-300 ul Solution B (3.0 M Tris pH 8.0, 1.24 % SDS, 5% sucrose, 0.1 M EDTA, water up to 50 ml with DEPC (diethyl pyrocarbonate, Sigma) freshly added at 60-80 ul per 5 ml solution) was added and incubated on ice for 10 min. After incubation, 1 volume of phenol (equilibrated, ultrapure, USB, Cleveland OH) was added and incubated on ice for 3 min then centrifuge 5 min in microcentrifuge

(Eppendorf 5415) at 16,000g. After centrifuging, a phenol/chloroform extraction was performed on the aqueous phase using 1 volume phenol/[chloroform/ iso-amyl alcohol (24:1)] (1:1). Purified DNA was then removed through ethanol precipitation (as described previously).

Probe Isolation

Plasmid pDCG9a, a plasmid containing most of *Drosophila melanogaster Gld* exon 4 (Cavener et al 1986) was digested with *Eco*RI and *Bam* HI and run on a 1.2% agarose gel in 1X TAE (0.04 M Tris, 0.114 % glacial acetic acid, 0.05 M EDTA pH 8.0). DEAE membranes (Scleicher & Schuler, Keene, NH) were inserted above and below the band of interest and the gel was run for 1/2 hr at 80v. The lower DEAE membrane was removed and placed in 100 ul high NET buffer (20 mM Tris, 1M NaCl, 0.1 mM EDTA pH 8.0) at 65 °C for one hour. The supernatant was then transferred to a new tube and the membrane was again washed in 20 ul high NET buffer, which was then added to the new tube. Ethidum bromide was removed from the supernatant by adding 1 volume of equilibrated (TE, pH 8.0) n-butanol. After mixing and centrifuging the tube the upper layer containing the n-butanol was removed. The remaining buffer containing the DNA was ethanol precipitated, pelted by centrifuging 15 min at 16,000 g, washed in 70 % ethanol and resuspended in TE.

GLD Staining

Larvae were rinsed in MQ water and bled into droplets of Grace's insect media on sterile microscope slides. Cells were allowed to settle for 30 minutes. Cells were then rinsed with Grace's and stained with and without glucose. The staining solution used was 10 mg/ml MTT (3-(4,5-dimethyl thiazolyl-2)-2,5- tetrazolium bromide, USB), 10 mg/ml PMS (phenazine methosulfate), and 0.6 M glucose in *Aedes* saline. Protected from light, the cells were stained for 30 min. After the staining reaction was completed, slides were rinsed twice in Graces and mounted with 7 % gelatin, 50 % glycerol and a coverslip. Slides were then visualized with a phase contrast and DIC at 400X within one hour of staining. Rapid observation of the slides is required since MTT forms large crystals which eventually lyse the target cells (Cox-Foster and Stehr 1994).

***In Situ* RNA Staining**

The cRNA probe was made from pDCG9a linearized with restriction enzymes and labeled using the Genius DIG (digoxigenin) nucleic acid labeling kit (Boehringer Mannheim, Germany, cat # 1175041). Probe was shortened by alkaline hydrolysis (labeled cRNA added to 1 vol DEPC treated water and 2 vol carbonate buffer { 60 mM Na_2CO_3}). Probe length was verified by running a sample of the probe on an agarose gel and performing an RNA blot subsequently visualized using DIG detection. *In situ* hybridization was conducted on cells bled into Grace's Insect Medium and allowed to settle for 30 min on poly-lysine treated slides, dipped and air dried. Cells were fixed in 4% paraformaldehyde/ 0.2% gluteraldehyde in *Aedes* saline for 30 min. After fixation,

the cells were rinsed in ethanol/glacial acetic acid (95:5) for 5 min and then rehydrated in a series of 70%, 50% and 30% ethanol in DEPC treated water for 10 min each. Control slides were treated with (100 ug/ml) RNase A (Sigma Chemicals), and 100 U/ml RNase Ti for 10 min at 37 °C in H_2O. All slides were then pre-hybridized (5X SSC, 5X Denhart's, 50% formamide, 4 mM EDTA, 1 % NA pyrophosphate, 250 ug/ml tRNA {Boehringer Mannheim}, 250 ug/ml denatured salmon sperm DNA {Sigma chemicals}) overnight at 37 °C. Hybridization was conducted overnight at 40 °C using probe diluted in hybridization fluid to a final concentration of 1 ug/ml. Hybridized cells were washed in 2X SSC with 0.1 % SDS at room temp for 5 min and twice at 50 °C for 15 min. After washing, cells were incubated in 2 % bovine serum in buffer #1 (0.075 M Tris-HCl, 0.14 M sodium chloride) for 3 hours and then in anti-DIG antibody conjugate diluted 1:5000 in buffer #2 (0.075 M Tris-HCl, 0.1 M sodium chloride; pH adjusted to 9.5, 0.05 M magnesium chloride). Color detection was done overnight using 3.5 % x-phosphate / 4.5% NBT in buffer #2. Slides were then washed for 5 min. in buffer #3 (100 mM Tris-HCl, pH 8.0; 1 mM EDTA) and mounted in Aquamount (Poly Sciences Inc., Warrington, PA). Glass cover slips were sealed with clear nail polish and the slides were observed at 400X using phase and DIC microscopy. Prior to sealing, all slides were maintained in a sealed humidified chamber to prevent drying.

Immunohistochemical staining

Insects were bled into Grace's Insect medium and hemocytes were allowed to settle for 30-60 min. Cells were then fixed in 4 % paraformaldyhide / 2 %

gluteraldehyde in Grace's insect media and then rinsed with A*edes* saline. Cells were then dehydrated in a series of 30 %, 50 %, and then 70 % ethanol in sterile H_2O and incubated in PBS/Tween/10 % gelatin (10 mM sodium phosphate, pH 7.5, 0.9 % NaCl, 0.1 % Tween 20, 10 % liquid fresh gelatin) for 40 min., followed by 40 min. in straight normal goat serum (Sigma Chemicals). Cells were then rinsed in PBS/Tween/1% gelatin and incubated with anti-GLD antibody for 60 min, rinsed 2X in PBS/Tween/1% gelatin and incubated for 60 min with biotinylated 2^o antibody (from Vecta-stain ABC kit, Vector Laboratories, Burlingame, CA). Cells were then incubated in ABC reagent (Vecta-stain ABC kit) for one hour, then in Vecta-red alkaline phosphate substrate (Vector Laboratories) (2.5 ml 100 mM Tris HCl, 50 ul reagent 1, 50 ul reagent 2, 50 ul reagent 3) for 30 min. Cells were then rinsed 2X with Tris-HCl, pH 8.2, and mounted using gelatin/glycerol mountant (Cox-Foster and Stehr 1994). Cells were visualized using fluorescent microscopy using rhodamine and florescein filters at 400X. Images were captured via video camera and stored on computer disc.

Double Filter Lifts

An *A. aegypti* genomic library was obtained from Dr. Anthony James, Department of Molecular Biology and Biochemistry, University of California. The library was produced using Lambda DASH II (Stratagene) vector and grown using XL-1 Blue MRA(2) cells (Stratagene). Lambda phage plaques were grown 8-12 hours on 0.7 % top LB media agarose (150 mm plates, 2,500 -3,000 pfu per plate) at 37 °C and then were chilled. Filters (Amersham, Hybond N+) were placed on plate for 30-60 sec and

then removed. A second filter for each plate was allowed to sit for 1-3 min. Filter orientation was marked by asymmetrical marking of filter and agar using a hypodermic needle and India ink. Filters were denatured (0.5 N NaOH, 1.5 M NaCl) for 1-5 min, neutralized (1.5 M NaCl, .5 M Tris-HCl pH 7.4) for 5 min., then rinsed in 2X SSC and UV cross linked in a Stratolinker 2400 (Stratagene, La Jolla, CA).

Filter Blotting

Radio-labeled ^{32}P and ^{33}P probes (Amersham Life Sciences) were constructed using the Random Prime Kit (Amersham Life Sciences) with 25 microcuries per reaction. Filters were pre-hybridized (6X SSC, 10 % Denhart's, 0.5% SDS, Herring sperm {final conc. 250 ug/ml} DNA, 50 % formamide) for 2 hours at 37 °C and hybridized overnight at 30-37 °C overnight. Filters were washed twice in 2X SSC at room temp and twice in 2X SSC at 40 °C. Film exposure varied from 6 hr to 3 days for ^{32}P and 3-7 days for ^{33}P depending upon probe activity. Film exposure for ^{32}P labeled filters was accelerated using intensifying screens (Dupont Chemicals) at -80 °C.

Phage Prep

Phage DNA preparations were conducted as per Sambrook et al. (1989). Overnight bacterial cultures were digested and poly-ethylene glycol (PEG) treated on ice for one hour. The mixture was centrifuged and the pellet resuspended in SM (0.58 % NaCl, 0.2 % $MgSO_4 \cdot 7\ H_2O$, 0.05 M Tris, pH 7.5; 0.01 % gelatin) and treated with

protease K. Phage DNA was then removed through ethanol precipitation, resuspended, PEG treated, peleted and resuspended in TE.

Plasmid Prep

Plasmid DNA preparation was conducted as described by Sambrook et al. (1989). Transformed bacteria were grown overnight in LB media and then centrifuged for 30 seconds. The pellet was resuspended in 50 ul (50 mM glucose, 25 mM Tris-Cl, pH 8.0; 10 mM EDTA, pH 8.0), then 200 ul (0.2 N NaOH, 1% SDS) was added and mixed. Then 150 ul ice cold (5 M potassium acetate, 11.5 % glacial acetic acid and 28.5 ml H_2O, pH 4.8) was added, and the tube was vortexed. After vortexing, the solution was incubated and then centrifuged at 16,000 g. The DNA was then purified from the solution using phenol/chloroform extraction, peleted and resuspended in 50 ulTE.

Southern Blot

Southern Blots were conducted using Hybond N (Amersham) nylon filters. DNA transfer efficiency was increased by nicking DNA using 0.2 M HCl prior to DNA transfer. Gels were then soaked in denaturing solution (1.5 M NaCl, 0.5 N NaOH) and neutralized (1M Tris pH 7.4, 1.5M NaCl). Blots were conducted overnight according to Sambrook et al. (1989), washed in 6X SSC, dried and UV cross-linked in a Stratalinker (Stratagene).

Transfection

E. coli XL1 Blue (Stratagene) were grown overnight in 0.4 % maltose in LB media at 37 °C. Bacteria were peleted (1 min, 4,000g) and resuspended in 0.01 M $MgSO_4$, mixed with phage plaques (isolated from *Aedes* library screens) which were suspended in SM. The mixture was incubated for 5 min at 37 °C then added to NZYCM media (1 % Nzamine, 0.5 % NaCl, 0.5 % Yeast extract, 0.1% casamine acid, 2 % $MgSO_4 \cdot 7\ H_2O$; pH 7.0) and grown overnight.

Sequencing

Sequencing reactions were conducted using the Thermo Sequenase radiolabled terminator cycle sequencing kit, (Amersham Life Science). DNA was radiolabled using the Sequenase kit and ddNTP ^{33}P. The reaction products were run on a polyacrylamide gel (8 % acrylamide, 8 M urea, 1X TBE) for approximately 3 1/2 hours. Gels were then fixed in 10 % ethanol, 10 % acetic acid solution for 1 hour then dried under heat and vacuum. Dried filters were then exposed on X-ray film for 2-4 days. Results were read manually at least twice to proofread for errors. Sequences were then compared against known sequences in Genbank using BLAST-N and BLAST-X searches.

Results

Is GLD Activity Present in *A. aegypti* Hemocytes?

The first experiments conducted were to determine the presence and activity of GLD in *A. aegypti* hemocytes. Previous work by Cox-Foster and Stehr (1994) had demonstrated that, in the presence of glucose, GLD activity in hemocytes can be detected as a color precipitate when stained with PMS and either MTT or NBT.

Hemocytes stained in the presence of glucose formed dark precipitate granules located in the cytoplasm, while non-glucose treated cells remained colorless, indicating that *A. aegypti* hemocytes did posses GLD activity (Fig. 2). The low cell counts of the larvae made differentiation of cell types difficult. However, stained cells exhibiting granule formation were larger and spread out, characteristics indicative of plasmatocytes. Stains with either MTT or NBT both produced colored reaction products, although, the reaction with MTT was much faster and more intense. However, the granules formed from MTT staining continued to grow and eventually destroyed the stained cells, necessitating the viewing of stained cells within one hour of staining.

Is *Gld* RNA Transcribed in Hemocytes?

In situ RNA hybridization was conducted on hemocytes obtained from larval mosquitoes. Due to the low number of hemocytes in each larva, several larvae were

bled onto each slide in an attempt to increase the number of hemocytes available for observation. Hemocytes were probed using a DIG-labeled RNA probe constructed from plasmid pDCG9a (Cavener et al. 1986) that encoded almost all of exon 4 of the *Drosophila melanogaster Gld* sequence.

Three series of *in situ* staining protocols were conducted (Fig 3). The probes used consisted of (1) an anti-sense probe (2) a sense probe, and (3) a second anti-sense probe used on cells that had been exposed to RNase A and RNase T1 prior to hybridization to serve as a negative control. Staining patterns demonstrated strong hybridization of anti-sense probes in the nucleus as well as the cytoplasm of hemocytes. Results from the sense probe showed some staining localized to the nucleus of the hemocytes. RNase-treated controls demonstrated weak staining. Determination of cell types exhibiting staining was difficult for these reactions due to the low cell counts, although most cells observed appear to be plasmatocytes.

Do *Drosophila* GLD Antibodies Detect GLD In *A. aegypti* Hemocytes?

Immunohistochemcal stains designed to determine if the GLD enzyme was present within *Aedes* hemocytes were performed (Fig. 4). As described previously, mosquito larvae were bled into a drop of Grace's insect medium, allowed to settle, and then fixed. Cells were then reacted with a rabbit anti-GLD (Cox-Foster and Stehr 1994).

Results from these slides demonstrate a strong fluorescence associated with what appear to be plasmatocytes. The strongest fluorescence was observed in what appears to be an aggregation of hemocyte types. Weak staining was sometimes observed in what may be granulocytes (smaller and significantly rounder cells) and almost no staining was observed in other cell types and debris present that were most likely the result of larval bleeding.

fig 2/3

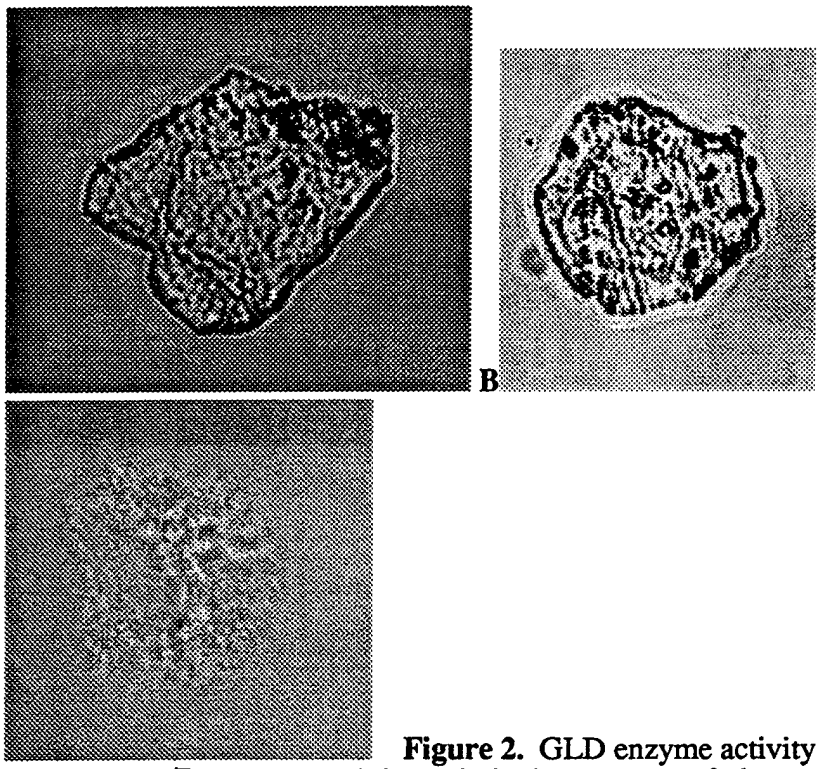

Figure 2. GLD enzyme activity staining in *Aedes* hemocytres. **A/B** Enzyme activity stain in the presence of glucose, note the large participate granules present. Fig **C** Enzyme activity stained without glucose

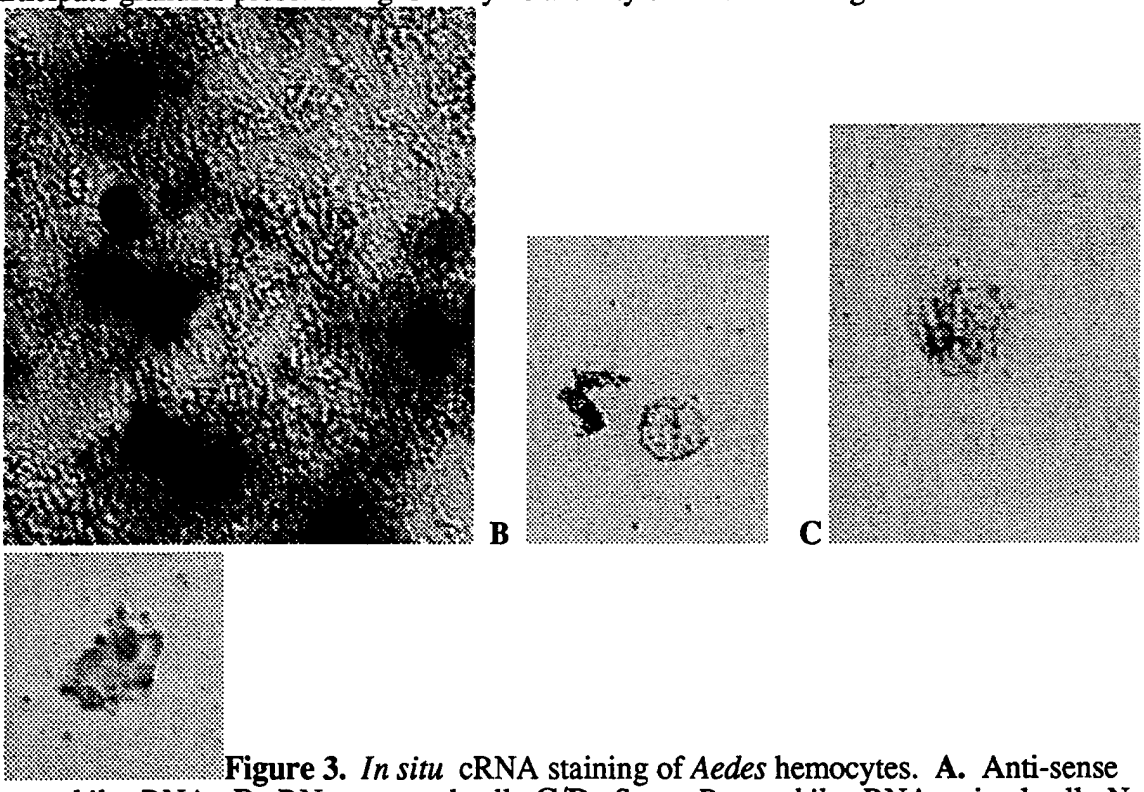

Figure 3. *In situ* cRNA staining of *Aedes* hemocytes. **A.** Anti-sense *Drosophila* cRNA. **B.** RNase treated cell. **C/D.** Sense *Drosophila* cRNA stained cell. Note the presence of stain within the nucleus.

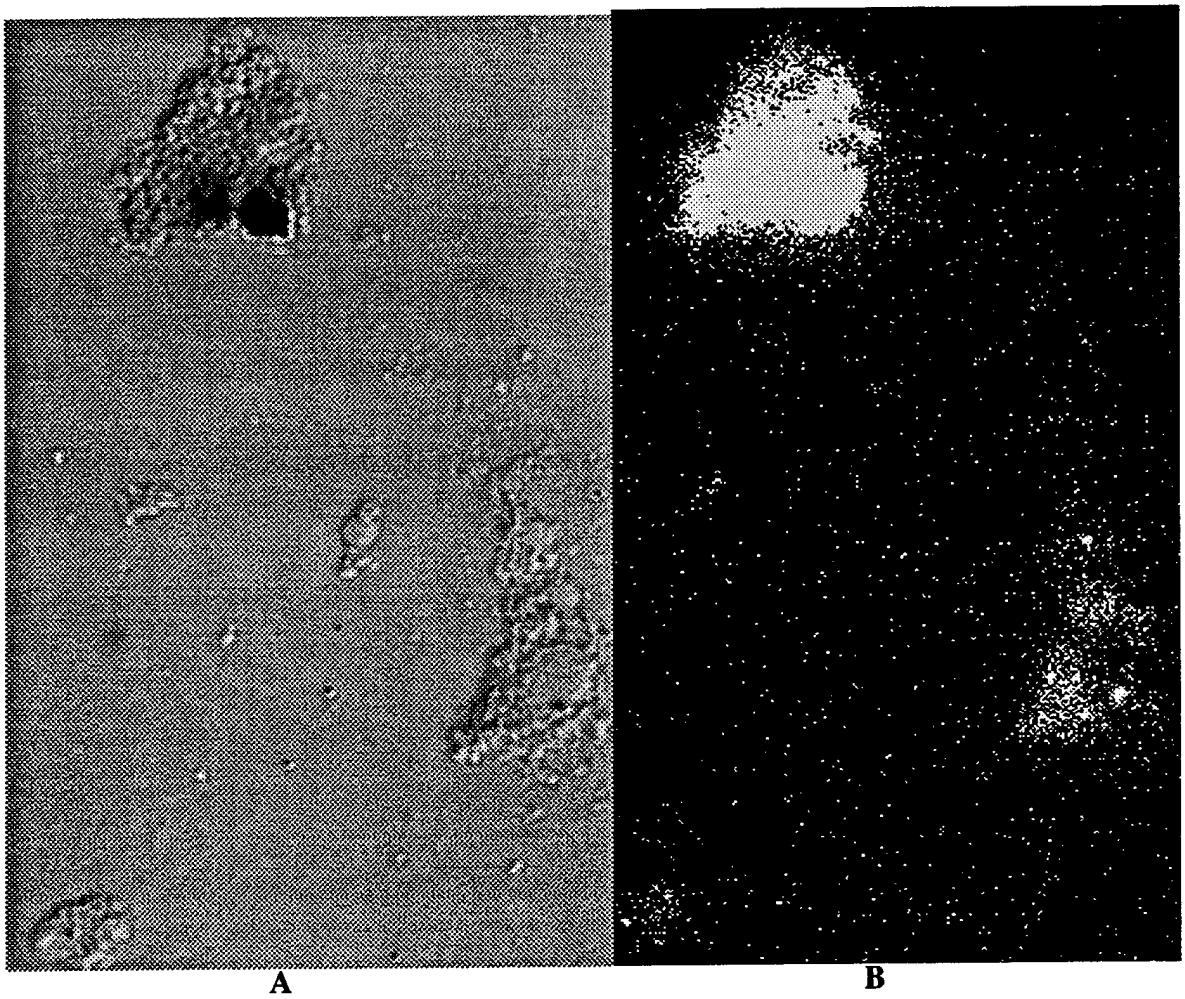

Figure 4. Florescent GLD antibody staining of *Aedes* hemocytes. **A**; DIC and **B**; florescent (rhodamine filter) images (400x) of *A. aegypti* hemocytes stained with *Drosophila* anti-GLD antibodies. Note extremely low signal observed from apparent granulocyte in lower left corner. Also not the apparent "shadow" created by the nucleus of the lower right plasmatocyte.

Blot Results

Having established the presence of GLD activity in larval hemocytes, several attempts were made to isolate the gene encoding *A. aegypti Gld.* DNA preparations were made from larvae as either a total nucleic acid isolation or DNA isolation. From these preparations a series of restriction digests, single and double, were conducted, run on agarose gels, and blotted onto nylon membranes. Several different probe attempts were made using both ^{32}P and chemiluminescent labeled probes. In all instances no definitive bands were observed in any lane containing genomic *A. aegypti* digests.

Also included as controls in these blots was genomic DNA isolated from *Drosophila* and *M. sexta* and digested with the same restriction enzymes used on the mosquito DNA. No definitive bands appeared in any genomic lane. Binding of the probe was shown to plasmid DNA from both *M. sexta* and *D. melanogaster* containing *Gld* sequences, and non-specific binding was not observed to Bluescript DNA. The positive results obtained from the slide series and the lack of detectable bands in both the *M. sexta* and *D. melanogaster* lane might indicate that, because *Gld* is a single copy gene, the number of copies of the gene present in the genomic digests was not high enough to show definitive binding.

Library Screens

In addition to DNA blots, an attempt was made to locate a *Gld* clone from a lambda phage library *(Aedes* genomic library). Duplicate filter lifts were prepared from

23 plates of lambda plaques (2,500-3,000 pfu/plate) and probed with ^{32}P radiolabled pDCG9a. Several potential positives were observed, which were subjected to secondary and tertiary screens. From these screens three likely candidate plaques were isolated. Similar binding patterns were not observed from non-responsive plaque isolates that served as controls after the initial screens were completed.

Each candidate plaque was then amplified through bacterial infection followed by phage isolation and DNA extraction. Phage DNA extracts were then cut by a series of restriction enzymes. The DNA was electrophoresed on agarose gels to verify the presence of an insert, and was then probed by DNA blot. Chemiluminescent plasmid pDCG9a probes were used to screen for the presence of *Gld* in any of the phage isolates. Results from this set of probes was negative with no apparent binding of the pDCG9a probe detected, however, binding was noted to *D. melanogaster* and *M. sexta* plasmid DNAs containing *Gld* sequences.

Subcloning reactions were also attempted using the most likely phage isolates. Subcloning was conducted using a Bluescript (Stratagene) plasmid for ligation and XL-1 Blue strain of *E. coli* (Stratagene) for growth. Selection was based upon blue/white colony growth. Self-ligation controls verified that antibiotic and color selection was working. Several colonies were obtained and amplified. Plasmid DNA was isolated and digested with restriction enzymes, blotted and probed. Results showed no specific binding in any lane containing subcloned DNA, however bands were observed in *D. melanogaster* and *M. sexta* sequence lanes.

Sequencing Reactions

Attempts were made to sequence using the Thermo Sequenase protocol (Amersham Life Science cat # US 79750). Sequencing of products generated from *Drosophila* genomic DNA produced matches to known *Drosophila Gld* sequences and validated the specificity of the PCR reaction and the sequencing reaction.

Sequencing reactions were attempted on both PCR products from the lambda isolate and genomic *Aedes* DNA preparations. Results of these reactions were varied and some were unreadable because of multiple bands present for each base pair, most likely due to an insufficiently pure DNA template. However some reactions did work and readable sequences were obtained from both the phage and genomic DNA products. The best sequence was generated from the *Aedes* genomic sample from which an approximately 250 base pair sequence was obtained, which was identical from both primer directions.

After the products were sequenced the sequences were matched through Genbank using BLAST N and BLAST X search protocols. Initial search results from the genomic and lambda isolate sequences produced several non-specific similarities to anonymous cosmid sequences and a variety of different gene products from many different species. A subsequent search of Genbank has indicated similarity of the genomic sequence with a transposon like sequence. The significance of this match is yet to be determined.

Discussion

The hypothesis examined by this work was that *A. aegypti* mosquitoes contained a gene homologous to *Drosophila Gld* and that GLD would be expressed in mosquito hemocytes. Evidence for the existence and activity of GLD within *Aedes* hemocytes was obtained using enzyme activity stains and immunohistochemical reactions. Evidence for a *Gld* gene in *Aedes* was generated by performing *in situ* cRNA reactions. Although the gene was not sequenced, the evidence presented here may be the first characterization of an immune associated factor within an *Aedes* hemocyte.

The results obtained from this set of experiments present strong evidence for the expression of GLD by *Aedes* hemocytes. Three different staining techniques, (enzyme activity, immunohistochemical, and RNA *in situ*) all demonstrate positive results for *Gld* presence and activity in *Aedes* hemocytes. Enzyme activity staining showed distinct precipitate in cells stained in the presence of glucose. Antibody reactions resulted in positive staining in what may be plasmatocytes, possibly indicating an expression pattern among cell types. The greatest intensity of staining for GLD activity observed was observed in plasmatocytes. Although cell-type specific staining has been demonstrated (Cox-Foster and Stehr 1994), the low number of *Aedes* hemocytes did not allow for a significant evaluation of differential cell staining. *In situ* cRNA hybridization detected expression of GLD mRNA throughout the cell body. Control experiments using sense strand cRNA showed staining isolated to the nucleus, while RNase-treated cells demonstrated weak staining.

The presence and function of GLD in *Aedes* hemocytes is not surprising considering that the presence and function of G*ld* has been demonstrated in several different species (Diana Cox-Foster, personal communication). In addition to several different species of *Drosophila*, GLD activity and protein has been detected in *Manduca sexta,* as well as arthropods as evolutionary removed from flies as crayfish. Therefore, the existence of GLD in mosquitoes, which are relatively closely related to *Drosophila* evolutionarily, is not unexpected.

In *M. sexta, Gld* expression appears to be limited primarily to the plasmatocytes and is not readily detected in other cell types. Similar claims can tentatively be made about GLD expression in *Aedes*; although, several technical problems prevent a definitive expression pattern from being determined at this time. The low hemocyte counts present in mosquitoes make it extremely difficult to isolate a sufficient amount of hemocytes for a significant study of cell types. In addition, insect hemocytes change morphology extremely rapidly in response to foreign material (glass slides) and different buffer conditions, making identification of cell types based upon morphology extremely subjective.

Several potential problems exist which would explain the inconclusive results obtained from the sequencing attempts. The sequence data based upon the lambda isolate may be based upon the incorrect library insert. If, during the initial screen, a false positive signal was selected, all subsequent work based upon that library insert would be in vain. A potential cause of failure of the genomic sequencing attempts may be the degenerate primers used. Random priming and extension could conceivably give

a semi-distinct band from a PCR reaction, but would generate a non-readable sequencing reaction; however, one readable sequence was generated. It also appears that the degenerate primers used may not be specific enough to sequence *Gld* from *Phormia*, a species more closely related to *Drosophila* than *Aedes*. It is most likely that this variation is even greater between mosquitoes and *Drosophila,* based upon phylogenetic trees. This variation might result in enough non-specific binding that sequencing reactions would be unreadable.

A modification of the primers used for sequencing might allow for the identification of the *Aedes Gld* gene. A current strategy being attempted is to "step" the primers to *Aedes* by using species more closely related to *Drosophila*, starting with *Phormia.* If *Phormia* can be sequenced, we will have *Gld* sequences available for a species beyond *Drosophila*. Comparison of these sequences should allow for a better evaluation of conserved sequences, hence more specific primers. In this manner we will continue to sequence closely related species until we reach *Aedes* and generate a successful sequence.

The existence of GLD in *Aedes* hemocytes identifies it as an potentially important component of the immune system, as is seen in *M. sexta* (Cox -Foster and Stehr, 1994). To date, little work has been done isolating specific factors from hemocytes in mosquitoes. Some work has been done in comparing expression patterns and localization of potential refractory molecules in different strains of mosquitoes (Paskewitz et al. 1989, Beerntsen et al. 1994). While significant data have been

obtained in these experiments, they focused on describing differences between refractory and susceptible mosquitoes while attempting to isolate refractory factors.

The identification of this immune component within *Aedes* hemocytes will open many areas of study. The experiments in this thesis were conducted upon larval mosquitoes. In nature, it is the adult female mosquito that expresses the refractory traits scientists are seeking to characterize. An important follow up study to this thesis will be a characterization of GLD expression throughout all mosquito developmental stages. Of particular interest will be the identification of GLD within adult mosquito hemocytes. If the enzyme does appear throughout the mosquito's adult life and is used in an immune capacity, it may represent an important weapon in disease control.

Another area to examine is determination of GLD content in the hemolymph of adult mosquitoes. In *M. sexta,* GLD was observed in cell-free hemolymph and was activated by immune challenge (Cox-Foster and Stehr 1994). In some mosquito species, it has been reported that the melanization reaction initially occurs without hemocyte involvement (Chen and Laurence 1985). Using the chemical pathway proposed in Figure 1 (Cox-Foster and Stehr 1994), GLD and phenoloxidase activities combine to produce the semiquinone radicals used to produce melanin. If GLD is discovered in the cell-free hemolymph of mosquitoes, it will be interesting to see if it plays a role in these acellular melanization reactions. GLD involvement in these reactions would offer a particularly potent weapon against vector-borne diseases.

Conclusions

In conclusion, evidence has been presented to show that *Gld* is present in *A. aegypti* hemocytes at a minimum. The data presented here are only the beginning of *Gld* characterization in mosquitoes. It may be that to detect *Gld* in *Aedes* the probes must be redesigned. Sequencing *Gld* sequences from species closer to *Drosophila* than *Aedes* will allow for the design of better probes. With more stringent probes the *Aedes Gld* gene will be identified.

The importance of understanding the insect immune system, and in particular the immune systems of vectors for infectious diseases is becoming greater each day. With the emerging drug resistant strains of malaria and pesticide resistant strains of mosquitoes a new control strategy is paramount. Better understanding of the genetics associated with the insect immune system and the natural refractoriness observed in some vector strains will play an integral part in the development of novel control measures.

The data presented in this work add to the overall understanding of the insect immune system. Continuing work in this area should elucidate the *Aedes Gld* gene. Full knowledge of the *Gld* gene in *Aedes* as well as the function of GLD will allow for better understanding of the mosquito immune system. With enough effort the knowledge will be obtained to understand the immune system, and the ability to manipulate the genetics behind the system will be obtained. When this happens

mankind will be well on the way to eliminating the vector-borne diseases that cause so much damage today.

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